Doctoral (PhD) theses

Studying the accumulation of materials with estrogenic effect on fields treated with dairy cow slurry and on plants (*Zea mays* L. *Lolium multiflorum* L.) on their several fenophases grown on those fields

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Mosonmagyaróvár
2022

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Theses

Studying the accumulation of materials with estrogenic effect on fields treated with dairy cow slurry and on plants (*Zea mays* L. *Lolium multiflorum* L.) on their several fenophases grown on those fields

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I. Introduction

The growing presence of Endocrine disrupting chemicals (EDCs) as micropollutants in our environment is more and more prevalent. They are very often of industrial origin (such as solvents of plastics), they may be of human origin (e.g. metabolits of contraceptives in the wastewater), but unfortunately their source may be the intensive agriculture at rising level as well. Slurry, appearing in intensive farms of animal husbandry as a side-product, contains not only valuable nutrients but also antibiotics, antiinflammatory pharmaceuticals and the above-mentioned EDCs that have adverse effects to the normal male reproductive functions the most. The adverse effects can affect aquatic and terrestrial wildlife alike.

Through spreading slurry in agriculural fields, EDCs can enter into the soil, where plants can take them up and, as a consequence, they can enter into the human food chain, even into humans themselves at the top of the food chain. The appearance of EDC compounds in natural acquifers, rivers and then in the drinking water is more and more widely known; however, the path of hormonally programmed intensive animal husbandy – slurry application – plant production – crop (i.e. fodder) with EDCs inside is not widely known at all. The thesis is intended to give an insight into that topic.

For this research, sampling was implemented on an intensive dairy cow farm in Pest county, Hungary, between 2017 and 2020. Samples were taken from the slurry storage pool on a quarterly basis. The estrogenic effects of the samples were measured with a holistic

(YES test) and an istrumental analytical (UHPLC-FLD) method. Results were compared to the reproduction data of the farm in order to find correlations between the use of hormonal injections and the level of EDC-content of the slurry using statistical methods. We measured the appearance and accumulation of compounds with estrogenic effect applying several testing methods.

II. Aims and hypotheses of the research

This research was carried out in Pest county, Hungary, and its aim is to study the appearance of the materials with estrogenic effect in the intensive cattle farming.

- Our major research aim was to identify and measure the EDCs in an intensive dairy cow farm.
- It was also our aim to study whether the *oestrus*-inducer products used at the cattle farm have estrogenic effect
- In order to reach our goals, we needed a validated *in vitro* method suitable for the analysis the EDCs. However, a ready-to-use, worked-out method was not available in the literature.
- The YES test (yeast assay) was suitable for the analysis of our samples and, therefore, we performed a methodology development and validation of it for slurry, soil and plant samples.
- For the most part, our research was conducted with using the *in vitro* YES test, but *in vivo* zebrafish tests and instumental analytical measurements applying UHPLC-FLD method were also performed as supplementary research.

- We studied the possible estrogenic content of our plant (*Lolium multiflorum* L., *Zea mays* L.), soil and slurry samples.
- We determined which parts of the plants and at which fenophases are able to accumulate materials with estrogenic effect.
- We studied whether long-term effect emerges at the same sites after a treatment with manure/slurry.
- How do the EDC-contents of the soils change between the slurry treatment and the harvest of the crop?
- Whether or not disorders do appear during the growth of the plant cultures?
- All the samples to-be-analysed were taken from real-life field conditions.

III. Methods applied

We conducted our research in an intensive dairy cow farm in Pest county. Among dairy cow stocks, the number of occasions of non-recognised *oestruses* is rising; for this reason, and for the continuous milk production, target-oriented breeding programs are employed. The advantage of these programs is that cows can be inseminated following a schedule. Their disadvantage is, though, that the level of estrogenic compounds rises in the slurry which, after applying the slurry on the fields, can appear either in the soil, or in different parts of the plants grown on them. On the one hand, it means the pollution of the natural environment (groundwater, wildlife), on the other hand, it also means the penetration of the esrogenic

compounds into the food chain through the harvested crop and fodder. Present topic has not been worked out in that broad sense and in such a detailed way.

Given that the present research is a path-finder work, most of the research methods used had to be adjusted first. The main research method was the YES-test (Yeast Estrogen Screen) worked out and adapted from ISO 19040-1:2018 international standard. Its foundation is that the genetically modified yeast (*Saccharomyces cerevisiae* BJ3505 strain), which contains human estrogenic receptor (hERα), in the presence of materials with estrogenic effect and CPRG (chlorophenolred-ß-D-galactopyranoside) substrate, gives a red product which is photometrable at 580 nm.

This method, which gives more holistic results (measures the total amount of estrogenic level) was supported with instrumental analytical measurements (ultra high performance liquid chromatography - fluorescent detector, UHPLC-FLD) for testing specific compounds in many cases. Compounds with estrogenic effects that were tested: E1, 17α-E2, 17ß-E2, EE2, E3. Using these methods we measured the estrogenic effects of the 5 *oestrus*-inductive hormonal injections (Alfaglandin, PGF, Dinolytic, Gonavet, Ovarelin), their 3 active substances (D-Phe6-gonadorelin, chloprostenol and dinoprost-trometamin) and 2 auxiliary materials (benzyl-alcohol and chlorocresol) applied at the farm.

Measurements covering all samples: YES-test

UHPLC-FLD method

Supplementary measurements/toxicological tests covering a limited number of samples:

- Zebrafish-test: transgenic zebrafish (*Brachydanio rerio* Tg(vtg mCherry) fluorencence test to reveal vitellogenin production)
- Germination tests (according to ISO 18763) to test phytotoxicity in cases of 5 plant species (*Lepidium sativum, Sinapis alba, Sorghum bicolor, Agropyron repens, Triticum aestivum x Secale cereale*)
- *Daphnia magna* 48-hour acute immobilisation test
- Algal growth inhibition test (*Pseudokirchneriella subcapitata*),
- *Azotobacter agile* test (MSZ21978-30:1988) for testing hazardous materials
- *Pseudomonas fluorescens* soil toxicity test (MSZ21470-88:1993)

IV. Results and Discussions

- According to our research, the EEQ values of the slurry were rising continuously, especially after the protocoll change in 2018.
- Estrogens tend to bind more strongly (up to 5 to 6 times) to the solid phase than to the supernatant.

- During present research, we studied 5 oestrus-inducer products (Ovarelin, Gonavet, PGF, Alfaglandin, Dinolytic). All of them showed estrogenic effect. Alfaglandin and PGF showed the highest estrogenic effect.
- During the cultivation of *Lolium sp*., we experienced that the EEQ value of the soil declines at first, then, after the N-fertilisation in the spring, it rises. The same tendency can be observed in the plant as well: after the nutrition treatment, the EEQ value rises significantly, then, following a condescending speed pattern of growing, reaches the highest value just before the harvest. That high EEQ value could be detected during the entire duration of the fermentation (about 200 days).
- The same tendencies could be observed during the cultivation of maize than in the case of *Lolium*. The differences are the following: the dose of the N-treatment was lower, after which the rise of the EEQ value was lower as well. While the EEQ value of the soil declined, that of the root was rising significantly. We could measure relatively lower values in the shoot and the grain crop. The fermented mass crop (silage) kept its estrogenic content.
- Using the method of Csenki *et al*. (2021), we performed a toxicological test employing transgenic zebrafish, with which we successfully detected estrogens from each slurry sample of four different dairy cow farms.
- During our research, 5 steroid estrogens (E1, ß-E2, α-E2, EE2, E3), 3 oestrus-inducer hormonal active substances (D-Phe6-

gonadorelin, chloprostenol, dinoprost-trometamine) and 2 auxiliary ingredients (chloprostenol, benzyl-alcohol) were measured with UHPLC technique from the slurry.

- We revealed that the root accumulates the EDCs more than the shoot, but the EDC content of the latter is still annoyingly high.
- In the toxicological tests, the highly diluted slurry did not show inhibiting effect, similarly to the less diluted but separated slurry samples.
- We did not experienc negative effects during the two soil toxicity tests and the Daphnia immobilisation test. By contrast, in cases of the algal tests, dilution as well as separation reduced the almost total initial inhibitory effect.
- In cases of 4 out of the 5 plant species in the germination tests (*Lepidium sativum, Sinapis alba, Sorghum bicolor, Agropyron repens, Triticum aestivum x Secale cereale*), only the 50 - 100 times dilution did not show inhibition. The toughest species turned out to be *Sorghum sp*.

V. New scientific results

1. Using several, different research methods, we successfully proved the **transmission** of EDCs and **their appearance in the resulting slurry, in the treated soil** and **in the roots, shoots and crop of the cultivated plants** grown on the treated soil. Present research approach is a new field for the science in Hungary and internationally alike.
2. Based on an international standard (ISO 19040-1:2018), we developed the *in vitro* YES test (Yeast Estrogen Screen) employing a **genetically modified yeast strain** (*Saccharomices cerevisiae* BJ3505). Furthermore, all samples were tested with an **instrumental analytical method** (UHPLC-FLD) for their estrogenic (E1, 17α-E2, 17ß-E2, EE2, E3) content. **Using a transgenic zebrafish strain** (Brachydanio rerio Tg(vtg mCherry) we proved the appearance of the EDCs in each slurry sample of 4 different cattle farms.
3. **Nitrogen fertilisation in the spring** raises the estrogenic content of the root and the shoot (stem plus leaves), not only in absolute numbers but also proportionately (per dry matter). We revealed that a kind of **a mechanism known in medical science as „blood-brain barrier" must exist** in the border of the root and the shoot, because only a fraction of an even extremely high EDC-level of the root

could appear in the shoot. But according to our measurements, the EDC-level appearing in the shoot is still annoyingly high from the point of view of the environment and human health.

4. We proved that in certain cases **ß-E2 can accumulate in very large amount in the shoot** as oppose to what the relevant literature claims. That accumulation can be observed more significantly in cases of slurry treatment than in manure treatment.
5. **Lime content, either in the soil or in the applied artificial fertiliser, can buffer (flatten) the rise of the hormonal content.** We quantified the amount of EDCs which was harvested per hectare. We proved that fermentation did not affect significantly the hormonal content of the fermented fodder, neither in the short, nor in the medium run; therefore, we cannot talk about a „half-life time".
6. **We proved that the compounds with estrogenic effect tend to bind more strogly to the solid particles** than to the liquid phase. In germination tests, both the unseparated slurry and the solid phase inhibited germination, even when the supernatant supported it in several cases. ***Sorghum bicolor*** turned out to be the toughest out of 5 plant species.

VI. Conclusions, Suggestions

- Several testing methods were employed to reveal the estrogenic footprint of the intensive milk production. Our primary method was the YES test, which gives holistic results.
- From the fact that we could not detect any out of the medicine active ingredients from the slurry, it can be concluded that all went through metabolization inside the animal.
- From the results of the YES test we came to the conclusion that it is worthwile calculating with a real and a visible hormonal effect. Alfaglandin kept its high, real hormonal effect throughout the process, even at high dilution. By contrast, the high hormonal effect of Gonavet was only a visible effect due to its synergistic auxiliary ingredient (chlorocresol). The EEQ values of Ovarelin and Dinolytic, which contain a different auxiliary ingredient (benzyl-alcohol) are *ab ovo* low and go below the detection limit quite quickly.
- Given that, as we pointd it out, EDCs tend to bind significantly more strongly to the solid than to the liquid phase of the slurry, at production scale with the use of a separator, we gain an inocent „irrigation water" and a compost raw material relatively concentrated for nutrients and EDCs.
- The EEQ value of the soil rises either after the use of slurry or manure. The accumulation of the EDCs in the root and the shoot of the fodder plant grown there can be abserved as well.

The level of that process accelerates significantly after the N-treatment in the spring. *Lolium* reacted for the treatments in a more dynamic way than silage maize.

- The EEQ values of the plants was the highest at the time of harvesting. That high EEQ value was not reduced by the conditions of the fermentation (acidic medium, anaerobic environment, the presence of lactic acid *bacteria*) even after 200 days of storage.
- The production cycle is closed by the fodder production. In the next production cycle, the animal will be given the stored mass fodder with its high EDC-content.
- Further research should be intended to reveal whether the EDCs appear in the milk and meat, coming from mass production.

Based on our results, we are going to give the following suggestions for the practice:

1. Based on our research, the de-hormonalisation of the fodder and the slurry starts already by choosing the right medicine. It is worthwhile choosing a medicine that reaches its high **visible hormonal effect** with the help of a synergistic auxiliary material.
2. Given, as we revealed, that the EDCs tend to bind more strongly to the solid phase of the slurry than to the supernatant, the use of the separator seems to be the most

practical and cost-effective tool of de-hormonalisation in the practice.

3. Our results showed that slurry, with or without separation alike, promoted the growth of *Sorghum bicolor;* therefore, fields just being treated with slurry are worthwhile utilised with that species at first.

VII. List of Publications

Journal articles in English:

Csenki, Zs., **Gubó, E**., Garai, E., Bakos, K., Sipos, D.K., Urbányi, B., Plutzer, J., (2022): Microinjection based zebrafish embryo test for the detection of estrogenic substances in slurry based irrigation water and its combined application with yeast estrogen screen; ***Agricultural Water Management***; 272; https://doi.org/10.1016/j.agwat.2022.107830,**(Q1, 6,5 IF)**.

Gubó, E., Kiss-Szarvák, I., Erdenebaatar, M., Gubó, R., Horváth, B., Szakál, P., Plutzer, J., (2021): Ecotoxicological investigations of milking cow slurry and changes of oestrogenic compounds in the solid and liquid phase; ***Energy, Ecology and Environment***; 7:97-110; https://doi.org/10.1007/s40974-021-00234-1, **(Q2, 3,8 IF)**.

Journal articles in Hungarian:

Gubó, E., Molnár, T., Szakál, P., Pordán-Háber, D., Bede-Fazeka, Á., Plutzer, J., (2022): Szarvasmarhatartó telepen alkalmazott

ivarzásindukáló hormonok megjelenése a hígtrágyában, ***Agrokémia és Talajtan***, pp 1-20, DOI: 10.1556/0088.2022.00116, **(Q4, 0,3 IF).**

Gubó, E., Szakál, P., Plutzer, J., (2019): Ösztrogének és ösztrogénhatású anyagok a növénytermesztésben; ***Agrokémia és Talajtan***; 68:385-401; https://doi.org/10.1556/0088.2019.00002,**(Q4, 0,4 IF)**.

International conferences:

Csenki, Zs., Garai, E., **Gubó, E**., Bakos, K., Vásárhelyi, E., Kánainé Sipos, D., Urbányi, B., Plutzer, J., (2020): Investigation of hormone-disrupting substances in slurry-based irrigation waters: the combined application of yeast and fish test; ***11th European Zebrafish Meeting***; 2020.10.26-27.

Hungarian conferences:

Gubó, E., Szakál, P., Plutzer, J., (2022): Szarvasmarha hígtrágya, talaj és növénykivonatok EDC (Endocrine Disrupting Compound) tartalmának meghatározása analitikai mérésekkel, ***XXV. Tavaszi Szél Konferencia***, **(Szekció 2. helyezés).**

Gubó, E., Szakál, P., (2022): Szarvasmarha hígtrágya, talaj és növénykivonatok EDC (Endocrine Disrupting Compound) tartalmának meghatározása analitikai mérésekkel, ***ÚNKP Konferncia 2021/2022***.

Csenki, Zs., **Gubó, E**., Garai, E., Bakos, K., Vásárhelyi, E., Kánainé Sipos, D., Plutzer, J., (2020): Hormonmoduláns anyagok hatásoldalról

történő vizsgálata hígtrágyaalapú öntözővizekben transzgenikus zebradánió (Daniorerio) és élesztő (Saccharomycescerevisiae) modell segítségével; ***X. Ökotoxikológiai Konferencia***.

Gubó, E., Szakál, P., (2021): Szarvasmarha hígtrágya EDC (Endocrine Disrupting Compound) tartalma és a telepen alkalmazott ivarzás indukáló gyógyszerek közötti összefüggések vizsgálata, ***ÚNKP Konferencia, 2020/2021***, pp 129-135.

Gubó, E., Szakál, P., Plutzer, J., (2020): Ösztrogénhatású anyagok vizsgálata a mezőgazdaságban az élesztőteszt (YES) segítségével, ***XXIII. Tavaszi Szél Konferencia***, pp 34. **(Szekció 3. helyezés)**

Gubó, E., Szakál, P., (2020): YES (Yeast Estrogen Screen) teszt adaptálása különböző talajminták EDC (Endocrine Disrupting Compound) meghatározására, ***ÚNKP Konferencia, 2019/2020***, pp 197-204.

Gubó, E., Szakál, P., Plutzer, J., (2019): Ösztrogének és ösztrogénhatású gyógyszerek jelenlétének vizsgálata tejelőszarvasmarha-telepek hígtrágyájában, **OTDK-Debrecen**. **(Szekció 1. helyezés)**

Gubó, E., Szakál, P., Plutzer, J., (2019): Ösztrogénhatású anyagok vizsgálata a növénytermesztésben: a hígtrágya-termőföld és takarmánynövény rendszerben, ***TOX'19 Tudományos Konferencia***, p 42.

Garai, E., **Gubó, E**., Csenki, Zs., Bakos, K., Balogh, E., Urbányi, B., Plutzer, J., (2018): Hormonhatású anyagok vizsgálata hígtrágya alapú

öntözővizekben: az élesztőteszt és halteszt együttes alkalmazása, ***TOX'18 Tudományos Konferencia***, p 56.

Gubó, E., Szakál, P., Plutzer, J., (2018): Ösztrogének és ösztrogénhatású gyógyszerek jelenlétének vizsgálata tejelő szarvasmarha-telepek hígtrágyájában, ***TOX'18 Tudományos Konferencia***, p 58.

Gubó, E., Szakál, P., Plutzer, J., (2018): Szarvasmarha hígtrágya ösztrogén tartalma szeparátor alkalmazása előtt és után, ***XXXVII. Óvári Tudományos Napok***, pp 411-420.

Gubó, E., Plutzer, J., Szakál, P., (2017): Ösztrogének és ösztrogénhatású gyógyszerek jelenlétének vizsgálata tejelőszarvasmarha-telepek hígtrágyájában, ***A Magyar Hidrológiai Társaság XXXV. Országos Vándorgyűlése,*** pp 1-20.

Additional publications:

Pordán-Háber, D., Kalocsai, R., Vona, V., **Gubó, E**., Szakál, T., (2021): A kritikusan fontos antibiotikumok (CIA) állategészségügyi alkalmazásának környezettoxikológiai jelentősége és hatása a víz-talaj kontinuumra, ***Acta Agronomica Óváriensis***.