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Fertilization of rye grass with carbamazepine, ibuprofen, and 17α-ethinylestradiol spiked urine – a greenhouse experiment

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Abstract
Urine is seen as an alternative fertilizer for agriculture as it contains relatively high concentrations of nitrogen, phosphorus, and potassium. But this usage of urine includes the risk of spreading pharmaceutical residues on to agricultural fields. In this study the uptake of carbamazepine, ibuprofen, and 17α-ethinylestradiol through rye grass fertilized with spiked male urine was investigated and analyzed by GC/MS. Plant matter production was not affected by the applied pharmaceutical concentrations but carbamazepine was taken up into roots (0.2 % of applied amount) and aerial plant parts (30 %). Concentrations of 17α-ethinylestradiol and ibuprofen could not be detected in plant tissue. It is assumed that they were biodegraded in soil.
No evaluation of toxic effects of pharmaceuticals ingested by humans through crops is possible at the moment from the findings of this research.

Keywords: pharmaceuticals, fertilization, urine, plant uptake

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1 Introduction

Urine, also called yellowwater, is discussed as an alternative fertilizer for agriculture as it contains relatively high concentrations of the macronutrients nitrogen, phosphorus, and potassium (Muskolus, 2008; Ganrot et al., 2007; Simons and Clemens, 2003; Vinnerås and Jönsson, 2002). But this usage of urine includes the risk of spreading pharmaceutical residues on to agricultural fields. Little is known on the fate of pharmaceuticals regarding their accumulation in soils, transfer to groundwater, and incorporation by plants in soils in the case of fairly high concentrations of pharmaceuticals as expected for urine. As today's wastewater treatment plants are not able to hold back pharmaceuticals effectively (Niederste-Hollenberg, 2003; Calmano et al., 2001), source separation of urine would protect surface waters from pharmaceuticals loads to some extends.

The uptake of pharmaceuticals in plants and the effects they exaggerate on plant physiology and development are of major interest especially for agricultural crops with regards to fertilisation with urine. Literature states that the pharmaceutical uptake of plants is correlated to the molecular weight of the pharmaceutical (Topp et al., 1986) and the octanol-water partition coefficient is looked at as a driving force for the uptake (Briggs et al., 1982).

Pharmaceuticals can affect plant growth when dosed in sufficient concentrations (Grote et al., 2004; von Euler, 1948). The question is, whether concentrations applied by urine fertilisation to fields are causing any adverse effects and how these effects would themselves manifest. Are pharmaceuticals taken up by these plants, in which concentrations, and into which plant parts?

2 Material & Methods

2.1 Selection of pharmaceuticals

The selected pharmaceuticals should fulfil several requirements. First, they should have been detected in German groundwater (so far 21 substances were listed in the database to be found in German groundwater). Additionally, they should differ with respect to log K_{OW}, molecular weight and their expected concentrations in AGU (see Table 1).

Table 1: Properties of the pharmaceuticals used in the plant experiments.

Pharmaceutical	Concentration in AGU[1] (mg l^{-1})			Indication[2]	log K_{OW}[3]	Molecular weight
	min	max	average			
CZ	0.00	0.12	0.058	Anti-epileptic	2.45 (at pH 7.4)	236.3
IBU	n.d.	1.27	0.844	Antiphlogistic, antirheumatic agent	2.41 ± 1.5 (at pH 7.4)	206.3
EE2	0.00	0.000047	0.000024	Sex hormone, Mestranol metabolite	3.67	296.4

n.d. = was not possible to determine this value due to lack of data.
[1] determined as described in Winker et al. (2008b), besides the external factors were not considered as they were not available at this time (State: Spring 2007).
[2] Arzneiverordnungs-Report 2004 (2004) and in case of EE2 additionally Orme et al. (1983)
[3] Hansch et al. (1995) and in case of EE2 Syracure Research Corporation (2004)

Consequently, three pharmaceuticals were selected: carbamazepine (CZ, CAS-N°. 298-46-4), ibuprofen (IBU, CAS-N°. 15687-27-1), and 17α-ethinylestradiol (EE2, CAS-N°. 57-63-6) (for their characteristics see Table 1). All three pharmaceuticals were purchased from Sigma-Aldrich: IBU, minimum 98 % GC; EE2, minimum 96 % HPLC, and CZ (no information on purity given by provider).

The urine used as fertiliser in the plant experiments was spiked with the average concentration of the three pharmaceuticals calculated for AGU according Winker et al. (2008b) further on referred to as "natural" (n) concentration level. Additionally, a higher level was used (referred to as "artificial" (a)): the ten-fold concentration in case of CZ and IBU and for EE2 a 40-fold concentration compared to "natural" concentrations in order to ensure the possibility of analytical detection. Beside the single pharmaceuticals, several combinations of pharmaceuticals were tested at the natural and artificial concentration level as well as a combination of all three substances.

2.2 Application of pharmaceuticals

The plant experiments were accomplished in cooperation with the Institute of Plant Nutrition of the University of Bonn from June to September 2007 in the greenhouse of the institute. A number of 64 "Kick-Brauckmann-pots" (height: 26 cm, diameter: 20 cm (Kick and Große-Brauckmann, 1961)) were filled with 9 kg air dried soil (Meckenheimer Krume; luvisol: 16 % clay, 77 % silt, 7 % sand (Schneider, 2005)). These pots contain a bottomless inner pot in a planter with drainage for leachate. In this experiment, the drainage was closed and all the water remained within the pot. Pots were connected to a micro-irrigation system (drop irrigation from Blunat) and adjusted to keep the soil moisture at 80 %. Soil moisture was controlled from time to time by weighing the pots. In week 23 (June 4-8, 2007), seeds of ryegrass (*Lolium perenne*) were seeded in rows into the pots (about 0.95 g seeds per pot). Germination and development of seedlings was regular.

Urine for fertilisation was collected from healthy males in bottles in the two weeks before application of urine spiked with the three pharmaceuticals designated as "UPmix". It was decided to use male urine to keep the hormone level as low as possible. None of the donors were under any medication. Urine used for the experiments showed average concentrations of macronutrients and TOC as well as average conductivity and pH (Tettenborn et al., 2007). After establishment and initial growth for a period of 2 weeks, the seedlings were treated with different UPmix. 250 ml liquid were added to each pot. Double pharmaceutical concentrations of AGU were added as pots obtained only half of the liquid planned to add. Amount of urine guaranteed an optimal supply for the growth period of three months.

Preparation and application of UPmix was carried out in three steps. First, pharmaceutical concentrates were prepared in the laboratory. The required concentration for the three repetitions was reached by dissolving the solid pharmaceutical in 500 µl methanol. The pharmaceutical-methanol (PM) solution was stored in the fridge at 5°C and transported in a cooling box. In the second step 750 ml of urine were measured with a 250 ml graduated beaker and the PM solution was added. The vial containing the PM solution was rinsed with 100 µl distilled water which was added to the UPmix as well. In the case of blanks, the same procedure was executed with the only difference being that the vial contained just 500 µl methanol without any pharmaceuticals. Afterwards, the UPmix was stirred with a glass rod, divided into three equal portions of 250 ml and applied to the pots. After each application, the full equipment was washed with distilled water before the next UPmix was prepared.

To keep pots with equal pharmaceutical additions as far away as possible from each other, the experiment was parted into three series: pot N°. 1-20, No. 21-40, and N°. 41-60. Pots N°. 61-64 were additional ones. In each section the same UPmix was only once applied. There were some variations in

measuring due to some remaining ml in the neck of the bottle. The experimental setup contained 2 blanks (pots only treated with urine and methanol) in each series as well as 4 pots completely untreated (no pharmaceuticals, no urine). The following morning plants of pots treated with UPmix did not show any differences to the untreated ones.

2.3 Harvest and sample preparation

Plants were cut seven times until harvest, the last time being September 9, 2007. Fresh and dry weight of the aerial plant parts cut was determined on each occasion. The experiment was terminated on September 17, 2007. Thereafter, all pots were completely emptied, root-soil content cut into several parts with a knife and roots were collected by hands for further analysis. Roots as well as plant parts close to soil, leftovers from cuttings, were washed with tap water and distilled water in sieves of pore sizes of 0.25 and 0.49 mm, separated from each other (roots and aerial plant parts left from cutting of leaves) and air dried. Afterwards, aerial plant parts were dried at 40°C for approx. 3 d until constant weight.

2.4 Analytical determination of pharmaceuticals

All pharmaceutical analyses were performed in the central laboratory of Hamburg University of Technology except analysis of urine.

Roots were cut with a cutting machine into fine parts and further ground in a coffee grinder. As only small amounts of root material was available (1.5 – 4 g DM), the entire material of one plant was used to prepare one sample. The grounded material was shaken for 2 h in a buffer solution of HCl and KCl (6.5 ml 0.2 M HCl and 25 ml 0.2 M KCl) at pH 2. Solid parts were filtered off by a pleated filter and the extract was subdued to solid-phase extraction with abselut Nexus cartridges (500 mg/12 ml, VARIAN). After washing the cartridges with rinsing water of the extraction bottles and then with few H_2O of analytical grade, the analytes were eluted with 5 ml methanol and the eluate was concentrated to a volume of 1 or 2 ml (roots) and 2 ml (aerial plant parts). Out of the three substances only carbamazepine could be determined, however as its decomposition product iminostilbene (CAS-N°. 256-96-2).

Table 2: Recovery rates, limit of detection and quantification of carbamazepine** and ibuprofen in roots and aerial plant parts.

Pharmaceutical	Recovery rate (%)	Limit of detection (µg kg^{-1} DM)*	Limit of quantification (µg kg^{-1} DM)*
Roots			
CZ**	56 - 61	10	20
IBU	67 - 98	20	30
Aerial plant parts			
CZ**	15 - 20	20	75
IBU	Not determined		

* Lowest recovery rate considered. ** Detected in form of iminostilbene.

The solutions were analysed by GC/MS (GC: Agilent 6890N; column: HP 5ms, ID 0.25mm film thickness, 0.25µm; MS: Agilent MSD 5975B, SIM, MS quadrupol temperature 150°C, MS source 230°C). The analysed peaks of each substance were verified by spiking the sample extracts with the respective pharmaceutical standard as the matrix of the extracts was problematic. Each sample was

analysed in duplicate, for recovery rates and LOQs see Table 2. Results are given to three significant figures.

EE2 was not detected. The reason was that uptake rates of plants were much lower than estimated during the preparation phase of the experiment and reported by Schneider (2005). Therefore, the chosen artificial concentration for EE2 was selected as too low.

2.5 Statistical evaluation of experimental results

Results of the pot experiments on rye grass were statistically evaluated with SPSS 15. A one-way ANOVA was accomplished as a one-way descriptive method in cooperation with a Student-Newman-Keuls procedure. α was set to be 0.05 to determine a significant difference between various treatments. For statistical analyses pots N°s. 49 and 51 were not included as due to a mistake during the experimental setup the applied UPmix of pot N°. 49 was not noted and N°. 51 was fertilised twice.
These two exposures (UPmix of IBU at artificial level and EE2 at natural level) were prepared a few days later in the additionally available pots N°. 61 and 62. Therefore, only two replicates could be integrated into statistics for the variant of CZ and IBU for the artificial level as it remained unclear which pot received two UPmixes.

3 Results & Discussion

3.1 Indirect effects of aerial plant parts towards pharmaceuticals

The growth of aerial plant matter (Figure 1) was identified for the entire 3 months experimental period. No visual effects were observed except Control 2 which received only irrigation water without nutrients. Aerial plant parts were smaller and thinner. The lack of fertilisation led to a large weight reduction. The overall fresh as well as dry matter of all plants fertilised with yellowwater did not show any effect irrespective of the kind and concentrations of added pharmaceuticals (Figure 1).

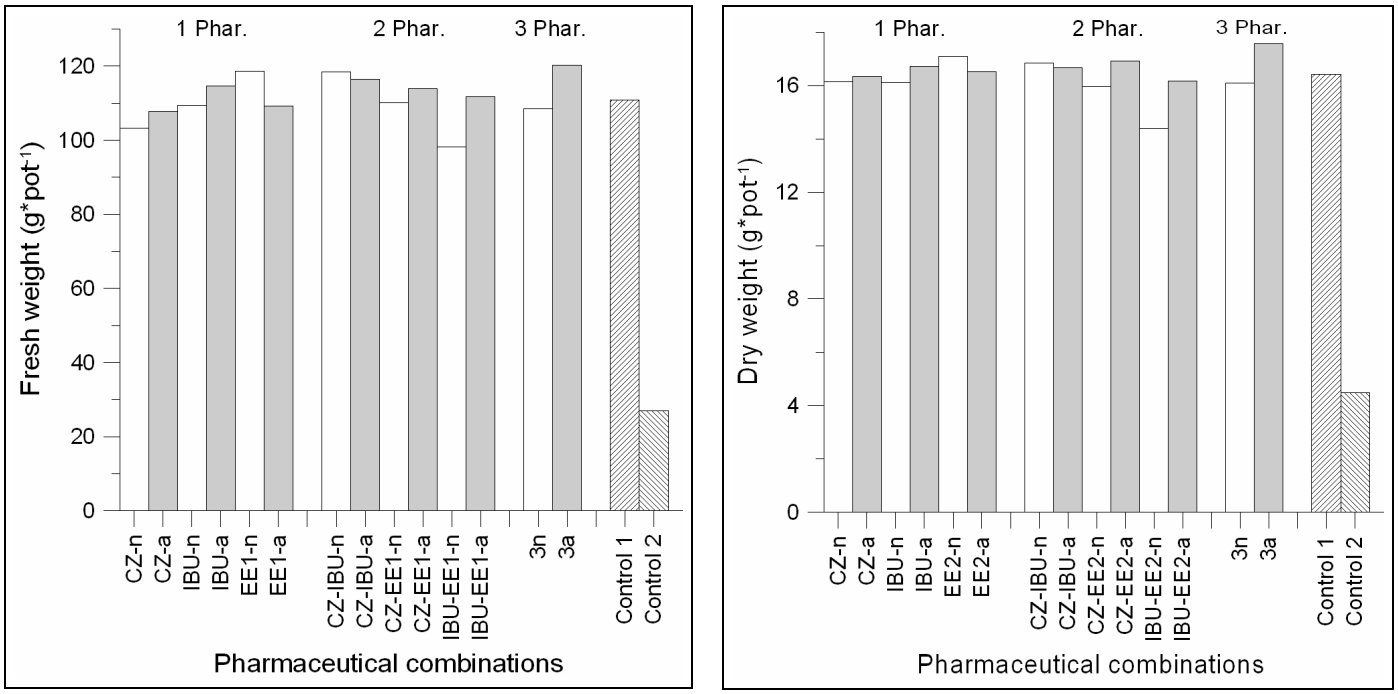

Figure 1: Overall fresh weight (left graph) and dry weight (right) of plant parts of rye grass determined during the full growth period. n = natural concentration (white bars), a = artificial concentration (grey bars). "Control 1" indicates plants treated with MeOH and urine, "Control 2" did not receive any application beside water; "3" is the designation for the combination of CZ, IBU, and EE2.

Statistical analysis confirmed these findings. One-way ANOVA, $P < 0.05$, showed a significant difference for the unfertilised control group for fresh and dry matter. Apart from this result, no significant differences were observed and the null hypothesis cannot be rejected which says that pharmaceutical applications do not affect the synthesis of plant matter at the tested concentrations. Moreover, no relationship was detected between the amount of harvested plant matter and the added doses of the particular pharmaceuticals applied disregarding the specific pharmaceuticals contained in the applied UPmix.

Besides the overall fresh and dry matter production, the dry matter production over the vegetation period was also determined. The factor of fresh weight to dry weight was only slightly varying between 6.01 (Control 2) and 7.03 (CZ-IBU-n). The pharmaceuticals did not have any effect on the course of the production of aerial plant parts during the growth period.

3.2 Uptake of carbamazepine and ibuprofen into roots and aerial plant parts

Due to the matrix of the plant material extract, detection of CZ and IBU were difficult. Only in the plants exposed to artificial concentrations, CZ could be quantified in the roots of rye grass. Similarly just CZ could be determined in the aerial plant parts and only its artificial concentrations could be quantified. CZ concentrations in the aerial parts of plants exposed to the natural CZ level (58 $\mu g\ l^{-1}$ in AGU) were in the range of the limit of quantification (75 $\mu g\ kg^{-1}$ DM). Detection of IBU in aerial plant parts was impossible due to the existing matrix effects.

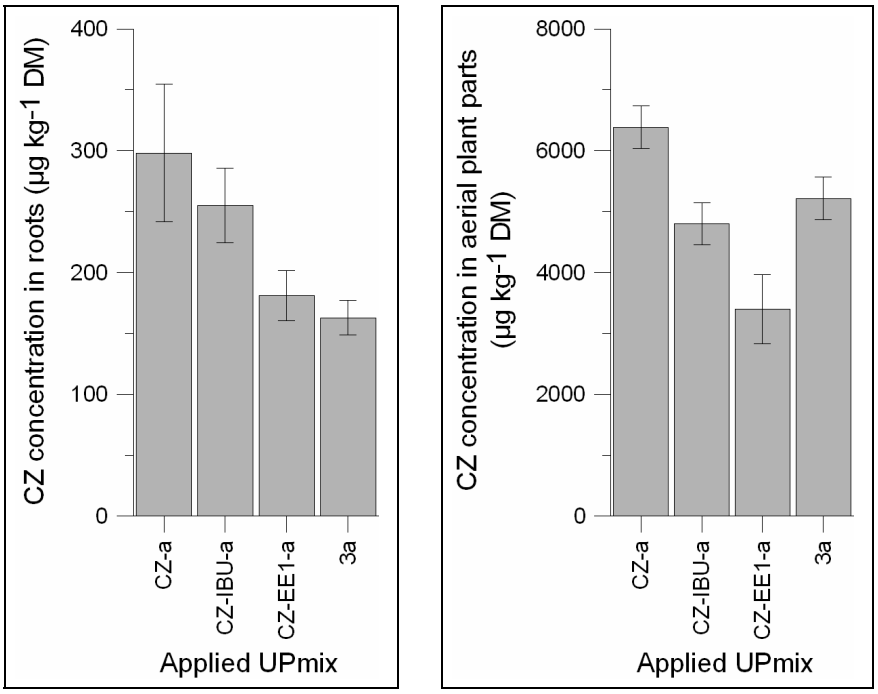

Figure 2: Mean concentrations of carbamazepine measured in samples of roots and aerial plant parts of rye grass after termination of the experiment at day 92 in µg kg^{-1} DM. Error bars show standard deviations among the three equally treated pots.

CZ concentrations in roots were between 131 µg kg^{-1} DM (a sample of CZ-EE1-a) and 426 µg kg^{-1} DM (a sample of CZ-a) with a mean concentration of 225 µg kg^{-1} DM while a tenfold concentration was reached in aerial plant parts (mean concentration was 4950 µg kg^{-1} DM; with a span of 2600 µg kg^{-1} DM (CZ-EE1-a) to 6950 µg kg^{-1} DM (CZ-a)). For completeness it has to be mentioned that instead of measuring CZ concentrations in roots in two pots the plant stocks were extracted and analysed as amount of root material was insufficient. As concentrations in the two samples of stocks were in the range of the two measurements within the roots of the same series (CZ-IBU-a: 243 µg kg^{-1} DM in the stock, 202 and 321 µg kg^{-1} DM in the roots; 3a: 131 µg kg^{-1} DM, 175 and 184 µg kg^{-1} DM), for these two pots the concentration in stock was assumed to mirror the mean concentration in roots (Figure 2).

Statistically relevant correlations between the uptake of CZ into roots and into aerial plant parts could not be determined. This might be a consequence of considerable coefficients of variation in the difficult matrix of plant extracts. Only when comparing the mean concentrations of the three pots of one UPmix, a weak correlation was observed. Overall, on average 0.21 % (between 0.12 and 0.40 %) of the total amount of CZ applied to each pot (under artificial conditions 290 µg per pot) was found in the roots of rye grass, but 30 % (between 15 and 42 %) in the aerial plant parts.

Relating these findings to the recovery of CZ and IBU in soil (Winker et al., 2008a), it can be assumed that IBU (neither detected in soil nor in plants) is not incorporated by plants due to its fast biodegradation in soil while CZ which stays in the soil for longer periods due to its recalcitrance remains available for plants for a much longer period and is thus transferred to the plants, especially to the aerial plant parts in the case of rye grass. It has to be pointed out that a discrepancy between the uptake rates into the aerial plant parts of natural and artificial concentrations existed. CZ

concentrations in the aerial plant parts exposed to the natural CZ level were in the range of the limit of quantification (75 µg kg^{-1} DM) and those exposed to artificial CZ levels showed an average concentration of 4950 µg kg^{-1} DM. At the same time in average 49% of the applied CZ was recovered in soils (Winker et al., 2008a) independently from the applied concentration (natural of artificial level).

This research does not consider the plants' behaviour towards pharmaceuticals i.e. a potential degradation in the rye grass during the growth period was not investigated. Moreover, no evaluation of potential toxic effects of pharmaceuticals ingested by humans with crops is possible at the moment with respect to the findings of this research.

4 Conclusion

- Exposure of rye grass to pharmaceuticals contained in urine at "natural" level (i.e. real as a consequence of medication calculated for AGU) as well as at higher concentrations did not affect the fresh and dry matter production during the growth period of three months neither for single pharmaceuticals, nor for the combination of CZ, IBU, and EE2.
- Only CZ was shown to be taken up by roots and aerial plant parts of rye grass. The CZ concentrations in aerial rye grass parts were in the range of 2500 to 7000 µg kg^{-1} DM and in roots 130 to 430 µg kg^{-1} DM This leads to the conclusion that only pharmaceuticals which are persistent in soil and are not biodegraded are transferred to plants in high concentrations. 30 % of CZ was found in aerial plant parts and 0.2 % in roots while IBU was below the limit of detection in roots (20 µg kg^{-1} DM).
- No evaluation of potential toxic effects of pharmaceuticals ingested by humans with crops is possible at the moment with respect to the findings of this research.

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