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Direct use of urine as fertilizer

potential risks of loading pharmaceuticals and hormones to field crops

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Zusammenfassung

Die ökologische Abwasserentsorgung, auch Ecosan genannt, ist eine alternative Methode, die die Nachteile der konventionellen, auf Wasser basierenden Abwassersysteme zu vermeiden versucht. Dieses Konzept, welches von der Funktionsfähigkeit der Ökosysteme sowie von geschlossenen Materialkreisläufen Gebrauch macht, beinhaltet verschiedene nachhaltige und umweltfreundliche Technologien, welche angemessene Entsorgungslösungen zu lokalen Gegebenheiten breitstellen kann. Der möglicherweise wichtigste Bestandteil der ökologischen Abwasserentsorgung ist die Erkenntnis, dass menschliche Auscheidungen und Haushaltsabwässer eine Rohstoffquelle (und nicht Abfälle) darstellen, welche die Möglichkeit einer Wiederverwendung bieten. Die vorliegende Studie befasst sich mit der Wiederverwertung menschlichen Urins als Düngemittel, sowie mit der Bewertung und Minimierung von Risiken, welche durch die Verbreitung der im Urin enthaltenen pharmazeutischen und hormonellen Rückstände auf landwirtschaftlichen Flächen auftreten könnten. Zu diesem Zweck wurde zum Einen eine Fallstudie in Bogotá, Kolumbien, durchgeführt, um sowohl die aktuellen sanitären Verhältnisse als auch die Einschränkungen und Stärken der angewandten ökologischen Abwasserentsorgungsstrategien in Stadtrand und ländlichen Gebieten Bogotás zu indentifizieren. Zudem wurde anhand einer kleinen Stichprobe häufig verwandte Arzeimittelgruppen ermittelt. Bei der Suche nach einer analytischen Methode, welche im Vergleich geringe Ausgaben für Ausrüstung und Einrichtung benötigt, entschieden wir uns für den Yeast Estrogen Screen (YES) Assay, um estrogene Stoffe in Pflanzenmaterial und Boden sowie im menschlichen Urin zu analysieren. Der YES Assay wurde in vorherigen Versuchsreihen als eine zuverlässige Methode zur Bestimmung der estrogenen Aktivität in Boden, Weizensamen und menschlichem Urin beschrieben. Jedoch beobachtete man auch Probleme mit dieser Methode, insbesondere in der Analyse von estrogenen Stoffen in bestimmten Pflanzenteilen wie Blätter, Stengel und Wurzel (z.b. Weizenwurzeln). Die vorliegende Studie konnte anhand des YES Essay das hohe und schnelle prozentuale Degradationspotential estrogener Verbindungen im Licht und Dunklen, sowie in hydroponischen Anbauversuchen nachweisen. Diese Ergebnisse deuten an, daβ ein geringes Risiko in der Anreicherung von Estrogen (βE2) auf landwirtschaftlichen Flächen besteht, da es zügig auf unterschiedlichen Wegen abgebaut werden kann (z.B. via Photodegradation und Biodegradation). Im Gegesatz dazu sollte man größere Aufmerksamkeit auf schwer abbaubare Verbindungen wie Carbamazepin (CBZ)legen, welches in dieser Studie in Blättern und Stengeln nachgewiesen wurde. Die Mengen dieses Stoffes in den verzehrbaren Pflanzenteilen (d.h. Weizenkerne und Sonnenblumensamen) waren jedoch relativ gering, und erreichten gemäβ unserer Ergebnisse keine therapeutisch relevanten Konzentrationen. Verapamil (VER) ist ein Arzeimittel, welches neuerdings auch als Mikroschadstoff in der Umwelt auftritt, noch nicht ausreichend untersucht wurde, und über

dessen Umweltverträglichkeit sehr wenig bekannt ist. Wir konnten nachweisen, dass es sich in Böden und Pflanzenteilen – und noch bedeutender – in eβbaren Pflanzenteilen aufgenommen wird. Obwohl die gefunden Mengen gering waren, gibt es weiteren Bedarf an Untersuchungen des Umweltverhaltens und des Abbaus dieses Stoffes in unterschiedlichen Ökosystemen (z.B. in Böden und Gewässern).

Summary

Ecological sanitation or Ecosan is an alternative approach to avoid the disadvantages of conventional water-based sanitation systems. This approach, based on ecosystem functioning and the closure of material flow cycles, includes different sustainable and environmental friendly technologies to provide appropriate sanitation solutions to specific local situations. Perhaps the most relevant aspect of ecological sanitation is that it recognizes human excreta and household water as resources (not as a waste), giving the opportunity for its re-use. This study focused on re-use of human urine as fertilizer and the identification and mitigation of risks of spreading pharmaceutical and hormone residues into the agricultural fields. For that purpose, a case study was performed in Bogotá, Colombia to understand the actual sanitation scenario as well as to identify the limitations and strengths of implemented Ecosan strategies in peri-urban and rural areas of Bogotá. Furthermore, a group of pharmaceuticals frequently used was identified for a small population group. In the search for an analytical tool that was cost effective, and also required low investment in facilities and equipment when compared to other analytical methods, it was decided to test the yeast estrogen screen assay (YES) for analysis of estrogenic compounds in soil and plant material as well as in human urine. The YES assay was a reliable analytical technique for finding estrogenic activity in soil, wheat grains and human urine. However, difficulties were encountered while analyzing estrogenic compounds in some plant tissues. The current research assessed the degradation potential of estrogenic compounds and discovered that estrogens contained in human urine can degrade under both light and dark conditions where exposure to light presented a slightly higher degradation rate. Degradation of 17β-estradiol (βE2) also occurred in the nutrient solution of a hydroponic system. The findings suggest that there is little risk of βE2 accumulation in agricultural fields since it is easily degraded via different pathways (e.g. photodegradation and biodegradation). Consequently, more attention should be paid to persistent compounds such as carbamazepine (CBZ) which was found in the plant leaves and stems. However, concentrations in the edible parts (wheat grains and sunflower seeds) were rather low, and according to the results did not reach concentrations for therapeutic use. Also, Verapamil (VER) which has not been frequently studied as an emergent micropollutant and very little is known about its environmental risk, was found in soils as well as in plants, and - most importantly it occurred in edible parts. Although the concentrations found were very low, there is the need to further investigate the effect and degradation potential of this compound in different environmental systems (e.g. soils and natural water bodies).

Erklärung (Declaration)

Ich versichere, dass ich diese Arbeit selbständig verfaßt habe, keine anderen Quellen und Hilfsmaterialen als die angegebenen benutzt und die Stellen der Arbeit, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen sind, kenntlich gemacht habe. Die Arbeit hat in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vorgelegen.

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List of abbreviations

~	approximately, about, around
°C	degree Celsius
µg	microgram
µL	microliter
µm	micrometer
ANOVA	Analysis of variance
APIs	Active pharmaceutical ingredients
ASU	alternative sanitation unit
BCF	bioconcentration factor
$C_{0\ \beta E2EQs}$	final concentration of βE2
$Ca\ (NO_3)_2$	calcium nitrate
Ca	Calcium
CAL-K_2O	Potassium oxide
CAL-P_2O_5	Phosphorus pentoxide
CBZ	carbamazepine
cm	centimeters
CO_2	Carbon dioxide
C_{org} %	Percentage organic carbon
CPRG	chlorophenol red-β-D-galactopyranoside
$CuCl_2$	Copper chloride
$C_{\beta E2EQs}$	βE2 concentration at time Zero
d	day
Da	Dalton
DL	detection limit
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DU	diluted human urine
DU-GIZ	diluted human urine from Deutsche *Gesellschaft für Internationale Zusammenarbeit*
DU-PH	diluted human urine from a private household
DW	dry weight
E1	estrone
E3	estriol
EC	electrical conductivity
Ecosan	ecological sanitation
EDCs	endocrine disrupting compounds
EDTA	Ethylenediaminetetraacetic acid
EE2	17α-ethinylestradiol
ELISA	enzyme-linked immunosorbent assay
ERs	estrogen receptors
Fe	Iron
$Fe_2(S0_4)_3$	Iron(III) sulfate
FW	fresh weight
g	gram
GC	gas chromatography
h	hour
H_2O	water
H_3BO_3	Boric acid
ha	hectare
HCl	Hydrogen chloride
HCO_3^-	Bicarbonate
hER	human estrogen receptor
HMR	7-hydroxymatairesinol
HPLC	high performance liquid chromatography
HRT	hormone replacement treatment
IUD	intra-uterine device
K	Potassium
K	reaction rate (d^{-1})
kg	kilogram
KH_2PO_4	Monopotassium phosphate
khz	kilohertz
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
L	liter
LAR	lariciresinol
LC	liquid chromatography
Ln	natural logarithm

log Kow	octanol/water partitioning coefficient
M	moles
m^3	cubic meter
MED	medioresinol
Mg	miligram
MgO	Magnessium oxide
$MgSO_4$	Magnesium Sulfate
mi	million
min	minute
mL	mililiter
mM	milimole
$MnCl_2$	Manganese(II) chloride
MS	mass spectrometry
mS cm^{-1}	millisiemens per centimeter
MTS	Meckenheimer topsoil
N	Nitrogen
n	sample size
$NaMoO_4$	Sodium molybdate
ND	not determined
ng	nanogram
$(NH_4)_2S0_4$	Ammonium sulfate
NH_2 (CO) NH_2	Urea
NH_4-N	ammonium-N
nm	nanometer
NO_3-	nitrate ion
N_{tot}	total Nitrogen
OH^-	hydroxide
P	Phosphorus
p	probability
PAH	polycyclic aromatic hydrocarbons
PCB	polychlorinated biphenyls
pH	hydrogen ion concentration
PIN	pinoresinol
PPCPs	pharmaceuticals and personal care products
ppm	parts per million
r	correlation coefficient
RH	Relative humidity
rpm	revolutions per minute
SEC	secoisolariciresinol
SEL	socio-economical level
SL	spinach leaves
SPE	solid phase extraction
SPM	standard pot substrate mix
STDEV	standard deviation
T	temperature
$t_{1/2}$	half-life or time in d to degrade half of initial concentration
UDDT	urine-diverting dry toilet
UDT	urine diversion toilet
UGG	Ultra Gradient Grade
UV-A	long ultraviolet radiation 320-400nm
UV-B	short ultraviolet radiation 290-320 nm
v/v	volume/volume
VER	verapamil
W	watt
w/v	weight / volume
WC	water closet
WG	wheat grains
YES	Yeast Estrogen Screen
ZnSO4	Zinc sulfate
βE2	17β-estradiol
βE2 EQs	βE2 equivalent value

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1 General introduction

1.1 Dirty history

Use of human excreta for fertilization of agricultural fields is an ancient practice. In Athens, "*the fields were enriched with the sewage of the city, brought by the main sewer to a large reservoir, and then led by brick-lined canals into the valley of the Cephisus River*" (Duran 1966). In many Asian countries (e.g., Japan, China, Korea, Vietnam), the use of human feces and urine was a common practice that still prevails in some regions (King 2004; Winblad and Kilama 1985). Mezoamerican civilizations such as the Aztecs in Mexico-Tenochtitlán used the human excreta for fertilizing their "chinampas" which were rectangular artificial islands made of mud and human waste that were used for agricultural purposes. Human excreta were also used for tanning leather and the city had a network of public latrines from which the human excreta was collected and it was eventually sold at the main market (Medina 2014). In most European cities, human excreta were collected in cesspools and privies. This material was called "night soil" since men that would pick it up and cart it away were allowed to do it only during night time. This material would then be sold afterwards to the farmers as manure (Jackson 2014).

Over time, it was realized that mismanagement of excreta could be a menace to public health. The tremendous growth of urban centers during the industrial revolution soon led to an overabundance of pollution in the streets which was frequently related to outbreaks of diseases. The introduction of water closets (WCs) or flush toilets connected to public sewage system in European cities was among others, a measure for improving the sanitation and avoiding outbreaks of cholera infections. These closed pipes would reduce human contact with excreta and other wastewaters, thus lessen the chance of infection (Jackson 2014).

Between the late 19th and 20th centuries, when big cities started to construct extensive sewer systems, sewage was initially discharged into surface waters eventually causing catastrophic pollution. In London for example, all the sewage was disposed into the Thames River. A cholera epidemic in the city in 1832, forced the sewer and sanitation authorities to reformulate the management of sewage. Similar measures were considered in other cities, as well. As a result, by the end of 19th century, many cities in Europe started to implement at least primary sewage treatment before discharge (Jackson 2014).

Since the implementation of WCs and the adoption of more hygienic practices in the Europe of the 19th century, public health has significantly improved, and these practices were extended to

other countries. However, the circumstances would eventually drive towards the end of recycling and massive use of nutrients from urine and feces in agriculture. During that time, farmers depended mostly on animal and human excreta for the fertilization of agricultural fields. Yet, the use of those products to grow food was in question, and gradually they were losing their economic value (Jackson 2014). These events most likely had an impact on the availability and quality of produce and consequently in the nutrition and health of low income people during the 19th and 20th centuries.

1.2 Sanitation in present

Nowadays societies rely upon centralized systems that are comprised of pit systems and water-based systems. Pit latrines are abundant in developing countries where sewage connections are not available. In these systems excreta is collected in a hole in the ground reducing the contact to humans. However, the pit is normally not sealed and infiltration of nutrients and pathogens can occur resulting in pollution of ground and surface waters. It can also produce bad odors and breeding of pests (e.g., flies, rats). Pits must be emptied after a period of time which is a very unpleasant job. It is costly and in many locations it is not safely managed. Therefore, users may prefer to build a new facility which requires more space and can be a drawback in densely populated areas (Esrey et al. 1998).

Water-based systems have been constructed on the assumption that sewage will be treated at the end of the pipe. However, according to WWAP (2015) over 80% of the wastewater worldwide does not receive any treatment at all. In the cases where wastewater is treated, the main sewerage system uses lots of water to carry other wastewaters and excreta to a high-tech treatment facility. These centralized systems present a number of challenges, including high energy and water requirements, large costs for construction and operation, and considerable maintenance requirements (USEPA 2004). Furthermore, a multistage wastewater treatment is not able to remove all of the pathogens and pollutants which are eventually released to the environment (Deblonde et al. 2011).

Current figures show that the world is still facing a lack of sanitation in developing and transition countries. According to UNICEF (2017) in 2015, 2.9 billion people had access to a "safely managed" sanitation service (a basic facility where excreta are disposed *in situ* or treated off-site). However, 2.3 billion people still lack basic sanitation service and among them almost 892 million people still practiced open defecation. The fact is that conventional water-based sanitation technologies may not be the right solution since they are too costly and governments do not have the resources and/or the political will to finance the system for the entire population.

Probably, the biggest disadvantage of centralized wastewater systems is that they do not facilitate the recycling of resources (e.g., water, soil nutrients). The valuable nutrients and trace elements contained in human excreta are not being reintroduced to the agricultural fields, and this is affecting soil fertility. Instead, most of the nutrients (e.g., Nitrogen) are altered or enter the water ecosystem causing pollution. Furthermore, the use of sewage sludge in agriculture is often restricted due to high contents of heavy metals and other hazardous substances originated from intermixing household and industrial wastewaters and rainwater from streets (USEPA 1995).

1.3 Water pollution

Occurrence of metals, bacteria, or ions like nitrates (NO_3-) and ammonia (NH_4+) in waters have been seen for several decades and their impact on human health and the environment are known and are subject to regulation and control (USEPA 2016). However, for the last three decades different studies have reported the occurrence of new compounds or "emerging pollutants" in wastewaters and surface waters. Those compounds do not have any regulation and in many cases effects on environment and human health are unknown or not very conclusive. In this group, Bisphenol A, phthalates, polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), pharmaceutical compounds, and compounds from personal care products (Sauvé and Desrosiers 2014) are included among others.

1.4 Pharmaceutical compounds in the environment and effects of exposure

The presence of pharmaceuticals and personal care products (PPCPs) as well as endocrine disrupting compounds (EDCs) in the environment has been a topic for many years now. These micropollutants enter the environment by different routes such as effluents of wastewater treatment plants and waste and landfill effluents (Kümmerer 2009). Several urban and rural areas, especially in developing countries, lack wastewater treatment. Consequently, household, industry, and hospital wastewater end up in the rivers. In addition, application of manure and/or biosolids to agricultural fields represents a potential source for inputs of pharmaceuticals into the environment.

"Active pharmaceutical ingredients (APIs) are molecules with different functionalities and physico-chemical and biological properties. They are developed and used because of the more or less specific biological activity" (Kümmerer 2009). Most pharmaceutical molecules are small molecules (molecular weight <900 Da) that are most likely to be absorbed into the body when taken orally. When those molecules are released into the environment, they are called

"micropollutants" because they are normally found in the $\mu g\ L^{-1}$ and $ng\ L^{-1}$ range (Kümmerer 2009).

Several studies have demonstrated the occurrence of pharmaceutical ingredients in wastewaters (Baker et al. 2013; Ternes 1998, 2001) ground and surface waters (Baker et al. 2013; Schwarzenbach et al. 2006), as well as in soils (Thiele-Bruhn 2003; Kinney et al. 2008). Pharmaceuticals and hormones have also been detected in the raw water used for drinking water production in Spain. The authors reported that despite the persistence of compounds (e.g., Phenytoin, atenolol, hydrochlorotiazide, sotalol and carbamazepine) for treatments, the removal was higher than 95% (Huerta-Fontela et al. 2011).

Concerns regarding the presence of PPCPs in the environment have been summarized (Kolpin et al. 2002) as: abnormal physiological processes and reproductive impairment, increased incidences of cancer, the development of antibiotic-resistant bacteria, and the potential for increased toxicity of chemical mixtures. Arnold et al. (2014) summarizes the effects of pharmaceuticals at individual and population levels of wild organisms. The most well known case has been the feminization of male fish exposed to EDCs in effluents of wastewater treatment plants. Bioaccumulation of PPCPs on earthworms has been demonstrated as well (Kinney et al. 2008). Jjemba (2002) reviewed some studies where controlled laboratory experiments show that exposure of plants to some pharmaceutical compounds contained in soils may affect plant development and growth. However, the author suggests that concentrations used in such experiments are unlikely to occur in the soil, therefore more realistic experimental conditions must be tested.

1.5 Ecological sanitation

Ecological sanitation or Ecosan was conceived as an alternative approach to avoid the disadvantages of conventional water-based sanitation systems. This approach is based on ecosystem functioning and the closure of material flow cycles, and includes different sustainable and environmental friendly technologies to provide appropriate sanitation solutions to specific local situations (Langergraber 2005).

According to Esrey et al. (1998), the adoption of ecological sanitation:

- Allows the prevention of diseases from the source. If properly managed and maintained, the systems should not smell or generate pests (e.g., rats, flies and other insects).
- Is affordable and has very low or no recurrent costs for operation and maintenance. The device itself can be relatively inexpensive and is not difficult to build.

- Protects water systems from contamination and consequently less water would be consumed. Most Ecosan toilets need no water, which makes it very attractive for areas with water scarcity.
- Allows and favors decentralized urban waste to resource management.
- Has the potential to increase sanitation coverage to the unserved population more quickly than any other system.
- Allows us to make use of the high fertilizer value of urine and to recover the resource value of feces for soil conditioning. Therefore, farmers could reduce their needs for expensive fertilizers.
- Assists to replenish soil nutrients to levels at which productivity will rise, improving nutrition of families.

1.6 Re-cycling of human urine as fertilizer

Perhaps the most relevant aspect of ecological sanitation is that it recognizes human excreta and household water as resources (not as a waste), giving the opportunity for its re-use (Werner et al. 2004). As already known by ancient cultures, both feces and urine are a good source of nutrients for crops, organic matter and energy.

Studies have found that urine contains up to 90% of the nitrogen, 50-65% of the phosphorus and 50-80% of the potassium of total excreta (Heinonen-Tanski and van Wijk-Sijbesma 2005). Those nutrients in urine are in water-soluble form which makes them easy to digest by microorganisms and readily available for plants or easily transformable for plant uptake (Kirchmann and Peterson 1995). Furthermore, urine is hygienically safe. Excreted urine by a healthy person has very few pathogens compared to feces (Madigan et al. 2003). These features make urine a very attractive fertilizer.

Re-use of urine as fertilizer requires source-separation from feces. This is performed by using a urine diversion toilet (UDT) or no mix toilet. Source-separation tends to reduce the input of nutrients that reach wastewater treatment plants, giving the opportunity for a much more hygienic handling and a better re-cycling of nutrients (Larsen et al. 2001). After separation, urine flows through separate pipes and to a storage tank that should be emptied periodically. Storage is an essential step for reducing fecal pathogens before application into the agricultural field. The pH, the temperature, and the storage time have significant influence on the hygienization of urine (Höglund et al. 1998, 2000, 2002). The authors concluded that ‘‘if stored at 20°C for at least 6 months, urine may be considered safe to use as a fertilizer for any crop’’ (Höglund et al., 2002).

Urine has been used for a large number of crops in different countries. Karak and Bhattacharyya (2011) presented a list of studies per country where human urine has been tested in different crops. Authors also highlight the effectiveness of human urine as fertilizer as compared to other manures and commercial mineral fertilizers.

1.7 Statement of the problem

Beside the positive features of human urine re-use as fertilizer, there are some negatives to consider. Approximately 70% of the metabolites from pharmaceutical compounds are excreted via urine (Curry and Whelpton 2017; Lienert et al. 2007a; Lienert et al. 2007b). In the interest of removal of such micropollutants from urine, Maurer et al. (2006) reviewed numerous studies. Some of the authors suggest that handling of PPCPs can be done by the separation from the nutrients and then by removal. Struvite precipitation, ammonia striping and nanofiltration have demonstrated to be effective for the separation of nutrients and micropollutants. Chemical oxidation appeared to be successful in eliminating micropollutants from urine. However, these treatment options require specialized equipment, an infrastructure, and knowledge.

Other chemical reactions resulting in the transformation and/or degradation of PPCPs and EDCs parent compounds may be experienced when reaching the environment. Exposure to sunlight and microorganisms may induce those reactions. Bacteria and fungi are well known for degrading such organic pollutants in water and soil environment (Kümmerer 2009). However, there is no sufficient information regarding the degradation of PPCPs in human urine.

There is evidence indicating that PPCPs and EDCs can be taken up by crops fertilized with wastewater or biosolids (Reshaw et al. 2008; Jones-Leep et al. 2010; Wu et al. 2010; Sabourin et al. 2012; Cortés et al. 2013; Dodgen et al. 2013). The uptake of PPCPs and EDCs have been detected in different sources of nutrients (e.g. animal manure, biosolids, wastewaster); either in soil (Boxall et al. 2006) or hydroponic systems (Herklotz et al. 2010; Shenker et al. 2010; Zhang et al. 2013). So far, little has been documented on PPCP and EDC residues that are excreted in urine and their transfer from soils to plants after field application (Winker et al. 2010).

1.8 Thesis objectives

To guarantee that human urine re-use as a fertilizer can be done routinely, the PPCPs and EDCs have to be mitigated to avoid undesirable contaminations in the food chain of humans and animals. To improve the safe use of human urine, this study aims to identify the risks throughout the process of recycling of urine as a fertilizer and to build a framework of risk reduction and/or prevention.

In this context the aims of this research are to:

- To survey the potential of human urine recycling and to investigate the scenario of pharmaceutical consumption in a case study conducted in the peri-urban and rural areas of Bogotá (Colombia).
- To assess the potential of YES assay as an analytical tool for screening estrogenicity on urine, soil and plant samples.
- To evaluate the degradation potential of naturally excreted estrogens in urine under different storage conditions.
- To investigate the potential of plant translocation of selected pharmaceutical compounds in hydroponic and soil based systems.

The thesis is comprised of six chapters. Chapter 1 presents the background information and the objectives of this investigation. Chapters 2 through 5 are structured as scientific papers. In Chapter 2, the case study in Bogotá, Colombia is presented. Chapter 3 presents the use of YES assay as an analysis tool of estrogenicity in human urine, soil and plant samples. Chapter 4 is dedicated to the potential of estrogens degradation in excreted urine during storage time before field application. Chapter 5 presents the experimental phase of translocation of pharmaceutical compounds 17β-estradiol (βE2), carbamazepine (CBZ) and verapamil (VER) in wheat and sunflowers under hydroponic and soil based crop systems. Chapter 6 gives an overall conclusion and recommendations.

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2 Ecological sanitation, re-use of human urine as fertilizer and pharmaceutical consumption: case study in Bogotá, Colombia

2.1 Introduction

Colombia is located in the north-western corner of South America, with coastlines in both the Atlantic and the Pacific oceans. Geographically, Colombia can be divided into five natural regions: Andean region, Pacific region, Caribbean region, Orinoquía region and Amazon region. The country has an annual precipitation of 3 billion m^3, making it a water rich country. It has nearly 2.5 times the global rainfall, which stands at 1.2 m^3 per year (The World Bank 2012).

Colombia is the third most populated country in Latin America after Brazil and Mexico. In 2017, its total population has been calculated as 49,547,337 (DANE). Most of the population is concentrated in the mountainous western portion of the country as well as the northern coastline, a large amount living in or near the capital city of Bogotá (The World Bank 2016a). According to the World Bank (2016b), the country´s 2016 GDP of USD 282.5 billion categorizes it as an upper-middle income.

Fresh water coverage is relatively high, at 88.5% for the nation as a whole. However in less populated areas the coverage is at 59%. Sanitation coverage is at 76%, with the majority of this being on-site sanitation, and with relatively little sewerage network outside the main urban centers (World Bank Group 2016a). Wastewater treatment in the country is less than 28% where most of this corresponds to urban wastewater (Salinas 2011). No more than 30% of the municipalities in the country have any wastewater treatment technology and this 30% is concentrated in municipalities with a population over 10 thousand inhabitants. In the urban centers, close to 40% of the wastewater is treated while the other 60% is disposed untreated to the rivers. From the municipalities with wastewater treatment systems, 1% corresponds to tertiary treatment, 70% to secondary treatment and 29% to primary treatment. Densely populated cities as Bogotá (~8 mi) and Cali (~3 mi) depend on primary treatment (Salinas 2011; Superservicios 2015).

Recognizing the poor coverage of water and sanitation in the rural areas of Colombia, the Ministry of Environment, Housing and Territorial Development (2010) has been working on the implementation of the regulatory frame “Chapter J: Alternative water and sanitation technologies for the rural sector” that contemplates different sustainable technologies to provide the rural sector with appropriate water, sanitation and waste management solutions to

specific local situations. Practices as rainwater harvesting, simplified and decentralized waterborne systems and dry sanitation, among others are introduced. Also, Ecosan is proposed as a practice to the reuse of human urine and feces as fertilizer and soil conditioner, respectively.

This case study performed in Bogotá is an approach to understanding the actual sanitation scenario as well as to identify the limitations and strengths of implemented Ecosan strategies in peri-urban and rural areas of Bogotá. Furthermore, it is an opportunity for the identification of pharmaceuticals that are frequently used and might be found in the environment.

2.2 Materials and methods

2.2.1 Location description

Bogotá D.C. is located in the southeastern part of the Bogotá savanna at an average altitude of 2640 m above sea level. The savanna is bordered to the east by the Eastern Cordillera of the Andes mountain range. The Eastern Hills (Cerros Orientales) run from south to north limiting city growth. The western city limit is the Bogotá River. The Sumapaz Páramo (moorland) borders the south and to the north Bogotá extends over the plateau up to the towns of Chía and Sopó.

The city is the largest and most populous city in Colombia with approximately 8 mi inhabitants (DANE 2018). Population in Bogotá is distributed in six socio-economical levels (SEL) (1 - very low; 2 - low; 3 - middle-low; 4 - middle; 5 - middle-high; 6 - high) which are based on income, access to education and access to health and social security system. According to this, 99% of the population of Bogotá is grouped into the very low (1), low (2) and middle-low (3). (Secretaría Distrital de Planeacion 2014). Bogotá is divided into 20 administrative localities (Figure 2.1).

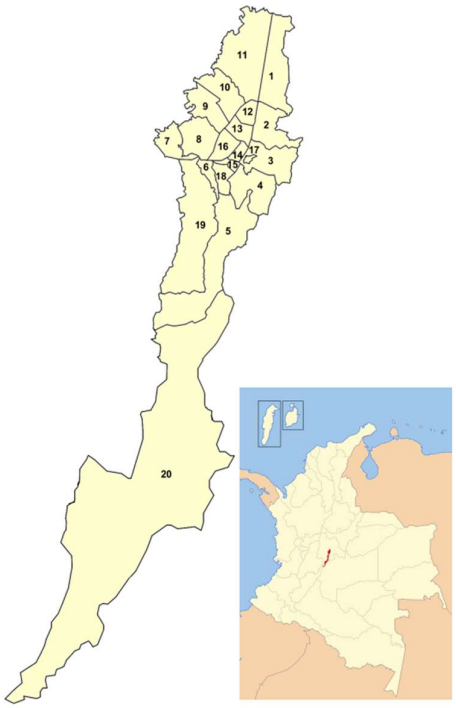
2.2.2 Ecosan and urine recycling in peri-urban and rural areas of Bogotá

Ecological sanitation has been implemented in Bogotá under the initiative: “Healthy Households” (“Hogares Saludables”) promoted by the district health care institution of Bogotá. This action aimed to increase the safe access to water and sanitation at regional level as mandated in one of the sustainable development goals.

Households with little access to water and sanitation facilities, and of socio-economical level (SEL) 1 and 2 located in the peri-urban and rural areas, and in informal economical activity or subsistence agriculture, were selected as beneficiaries for the installation of the “Alternative Sanitation Units (ASUs)” (Unidades Alternativas de Saneamiento). Other aspects like origin,

forced immigration and health issues due to lack of basic sanitation were also considered in the selection.

Every ASU included the installation of a water filtering system for human consumption, a urine-diverting dry toilet (UDDT), a rain water collection system, and a biofilter or constructed wetland with a fat separator to treat gray water. Thirty-two ASUs were installed in 2008 in the peri-urban and rural areas of the localities: Chapinero, Usaquen, Suba, Ciudad Bolivar and Usme (view locations in Figure 1). Two years later, only 15 ASUs were functioning and by 2013 only six households kept the units. In this study three ASUs are shown: one in Suba, one in Ciudad Bolivar and one in Usme. Every facility was photographically documented and observations about the management were made. ASU household members were also surveyed for the analysis of consumption and disposal of pharmaceuticals.



1 Usaquén
2 Chapinero
3 Santa Fe
4 San Cristóbal
5 Usme
6 Tunjueluito
7 Bosa
8 Kennedy
9 Fontibón
10 Engativá
11 Suba
12 Barrios Unidos
13 Teusaquillo
14 Los Mártires
15 Antonio Nariño
16 Puente Aranda
17 La Candelaria
18 Rafael Uribe Uribe
19 Ciudad Bolivar
20 Sumapaz

Figure 2.1. Map of Bogotá and the administrative localities.
Numbers in the map refer to each locality in the list. (Source: Wikipedia)

2.2.3 Analysis of consumption and disposal of pharmaceuticals

Data was collected through a survey. Initially it was intended to collect data about annual purchased pharmaceuticals from the local pharmacies. However, the access to that information required an authorization of national controlling entity, and due to time constraints, it was not possible to obtain. Therefore, data was collected only at the household level.

Surveys were made in a population group of the locality of Suba, located at the northwest part of the city (Figure 2.1, Number 11 in the map). Suba has an area of 10,056 ha, where 6,271 ha are urban soil and 3,785 ha are rural soil. By 2011, the total population of Suba was calculated to be approximately 1 mi inhabitants representing 14% of the population of Bogotá. (Secretaría Distrital de Planeacion 2014).

Two hundred households have been selected based on criteria established by the public health institution of Suba (Secretaría de Salud Publica). The criteria of selection are related to economical income and life quality standards as indicators of access to medical services (e.g. access to public or private health providers or no access at all) and indicators related to basic health practices at home (e.g. hygiene, waste management, pest management). Interviews were made face-to-face and the interviewed household member was always an adult (older than 18 years). The questionnaire was composed of 18 questions divided into six sections. The subjects treated in every section are detailed in Table 2.1 and the complete questionnaire is found in Appendix 1.

Table 2.1. Sections and respective subjects directed in the survey

Section	Subject
1	General household information: No. of members, gender, age, education level
2	Preferred options of illness treatment in the family
3	Access to pharmaceuticals
4	Intake of pharmaceuticals: Self-medication and frequency of consumption of analgesics and anti-gripals Chronic medical conditions, permanent medication (API*, concentration, doses)
5	Methods of pharmaceuticals disposal
6	Section for women: consumption of contraceptives and hormones for HRT**

*API Active Pharmaceutical Ingredient
**Hormone Replacement Treatment

2.3 Results

2.3.1 Ecosan and urine recycling in peri-urban and rural areas of Bogotá

In this study, three ASUs were visited: one in Suba, one in Usme and one in Ciudad Bolivar. There were two more units available. For one unit, the household owner was not available. The other unit was thought to be active but during the visit we realized the owners had recently uninstalled the diverting toilet and replaced it by a flush toilet. It was not possible to talk to the responsible person.

Alternative sanitation unit in Usme

Figure 2.2. View of the household in Usme and its terrain.

The unit was installed in the rural area of Usme. This is located at the south east of Bogotá (See Figure 2.1, Number 5 in the map). 86% of this locality is of rural soil dedicated to agriculture. Most of the agricultural products go to the city. This family owns a 2 ha farm where potatoes are mostly grown and they have a few cows for milk production (Figure 2.2). This five-member family was very satisfied with the use and the functionality of the diverting toilet system. The collected urine and feces are stored for at least 8 months before applying to the field (Figure 2.3). When applying, the family first divides the amount of urine and feces into different tanks. Due to the sloping terrain, they let the tanks roll from the top of the field and spread the material. After spreading, they allow the material to settle into the soil over a couple of weeks. Afterwards, they plough using animals. They also make use of the biofilter (Figure 2.4) where the filtered water runs into the field. Family members are in good health. However, one of them takes daily doses of enalapril, omeprazol, acetyl salicylic acid and folic acid.

Figure 2.3. View of toilet closure in Usme.

Left: under the closure floor, with an outside door are the storage tanks for the feces and urine collection. **Middle:** diverting dry toilet. Right: black tank collects the feces and the white canister behind collects the urine.

Figure 2.4. View of biofilter.

Left: Biofilter was installed outside the house area. **Right:** filtrated gray water outlet to the cropping field.

Alternative sanitation unit in Suba

This ASU was installed in the rural area of Suba. In this locality, 62% consists of urban soil and 38% of rural soil. Of the rural soil, 14% is protected and corresponds to wetland which is one of the most important ecosystems of Bogotá (Secretaría Distrital de Planeacion 2009). The two member family owns a 0.5 ha plot where they have their own vegetable garden and grow onions for selling at the local market. The members were very satisfied with the functioning of the diverting toilet. They have followed exact instructions for the use and the maintenance of the facility. Instructions for toilet maintenance always hang by the door (Figure 2.5). They have been

applying the collected urine and composted feces (Figure 2.6) into the field. So far, they have not applied any additional chemical fertilizers to the soil.

Figure 2.5. View of toilet closure.

Left: front of the toilet closure, besides is the shower cabinet and over the shower is the rain water collection tank; **Middle:** diverting dry toilet; **Right:** urinal. (Photos: Elkin Andres Peña)

Figure 2.6. Separation of urine and feces.

Left: Owner is showing the urine (blue) and feces (black) collection tanks; **Right:** composted feces are stored in sacs for future use. (Photos: Elkin Andres Peña)

Before the ASU was installed, the members have frequently experienced gastroenteritis, most probably caused by parasitic, viral or bacterial infection due to the very low quality of the water they were using. Their health improved once a drinking water filtration system was installed. In general, members are all in good health and they consume only homeopathic medicine.

Alternative sanitation unit in Ciudad Bolivar

Ciudad Bolivar is located at the south-west of Bogotá (see Figure 1, Number 19 in the map). This locality is one of the most densely populated in Bogotá (Figure 2.7) and it represents almost 10% of the city population. Households have the lowest SEL (1 and 2) and have poor access to water, sanitation and sewage (Figure 2.8). In this locality, 26% is urban soil and 74% is rural soil. The majority of rural soil is a conservation zone. Most of the urbanization was developed in abandoned quarries that were available in the 1950´s (Secretaría Distrital de Planeacion 2009).

Figure 2.7. Panoramic view of Ciudad Bolivar landscape

Figure 2.8. Rain water and wastewaters flow down the hill in the absence of sewage systems.

The ASU was installed in the community garden of one of the neighborhoods in Ciudad Bolivar (figure 2.9). The community garden serves people growing different kinds of vegetables and learning gardening practices for implementation at their own home-garden. The diverting toilet has been used mostly by men. Women do not feel very comfortable using it. The urine is left in containers for at least 6 months and afterwards it is diluted in order to spread into the planting

beds (Figure 2.10). According to the people using urine as a fertilizer, it has been of great help such that they do not have to collect much money to buy fertilizers. However, sometimes there is not enough urine because not many people are contributing.

Figure 2.9. View of toilet closure in Ciudad Bolivar.
Left: Toilet closure. **Right:** Dark green pot covered with a piece of light wood is the diversion toilet collecting only urine. Canisters are located under the building.

Figure 2.10. Overview of community garden in Ciudad Bolivar.

Upper left: empty field that has been irrigated with urine collected from the separation toilet two weeks before the picture was made. **Upper right:** maize and beans irrigated with urine. **Lower left and right:** Vegetables grown with different kinds of composts.

2.3.2 Analysis of consumption of pharmaceuticals by survey

Section 1: Number of household members, gender, age and education level

Out of 200 households, a total of 96 were interviewed from a total population of 370 individuals. The households were distributed in 3 different SELs as 43%, 35% and 22% in levels 2, 3 and 4 respectively. Due to security restrictions, absence of people at home and unwillingness to answer, it was not possible to collect information from households of socio-economical levels 1 (very-low), 5 (middle-high) and 6 (high). In general, households were composed from 1 to 10 members. However, households of 4, 3 and 5 members were more frequent (24%, 21% and 18% respectively). Age distribution of total population interviewed and level of education is shown in Figure 2.11.

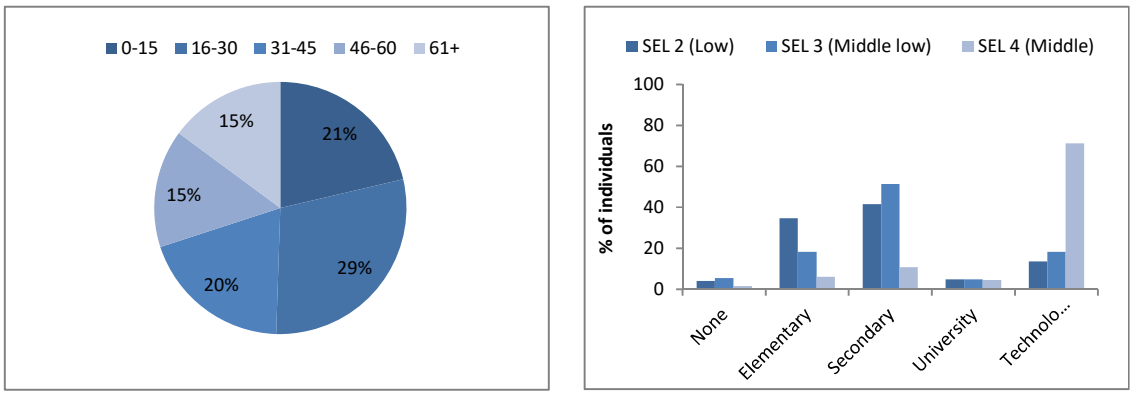


Figure 2.11. Age distribution of total population surveyed and level of education per SEL.

Section 2: Illness treatments

In the case of illness, most of the households (41%) prefer to go to the doctor for prescribed medicine, if necessary, while 25% of the surveyed households preferred homemade treatments and 11% favored self medication. When homemade treatments or self medication do not work, household members would go to the doctor.

Section 3: Access to pharmaceuticals

Close to 50% of the surveyed households acquire their medicine at a pharmacy and almost 45% can obtain medications through the medical center. A smaller number get their medicine from outside the country, especially when the medications are too expensive.

Section 4: Intake of pharmaceuticals

According to the collected data, in 52 of 96 households, there is at least one person under permanent medication. The distribution of permanent medicament consumption per age ranges is shown in figure 2.12 (left). The highest amount of permanent medicated individuals is in the age group greater than 61 years old. Antihypertensives are the most prescribed pharmaceuticals in the surveyed population group (Figure 2.12, right). The active pharmaceutical ingredients (API) of the most consumed medicines are presented in Table 2.2.

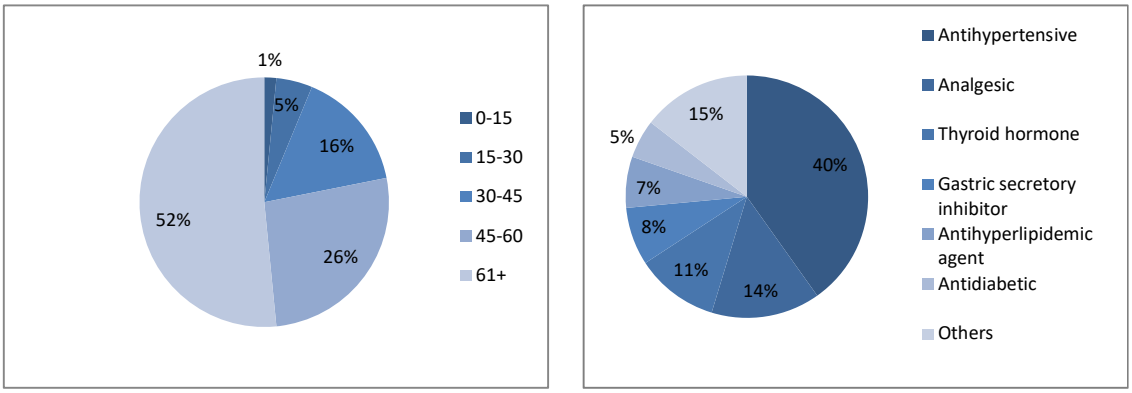


Figure 2.12. Permanent medication in the surveyed group.

Left: Distribution of permanent medicated individuals by age range. **Right:** Distribution of permanent consumed pharmaceuticals by therapeutic use.

Table 2.2. Active Pharmaceutical Ingredient (API) of the most consumed medicines.

APIs corresponding to each therapeutic use are listed from the most, to the least frequently used.

Therapeutic use	Active Pharmaceutical Ingredient
Antihypertensive	Enalapril maleate
	Losartan potassium
	Hydrochlorothiazide
	Metoprolol tartrate
	Verapamil hydrochloride
Analgesic (antipyretic, antiinflamatory)	Acetylsalicylic acid
	Acetaminophen
	Meloxicam
	Diclofenac potassium / sodium
Thyroid hormone	Levothyroxine
Gastric secretory inhibitor	Omeprazole
Antihyperlipidemic agent	Lovastatin
Antidiabetic	Human insulin
	Metformin hydrochloride
	Glibenclamide
	Sitagliptin phosphate

Most over the counter medication consumed by the households were found to be analgesics, decongestants, and antihistaminics (Figure 2.13).

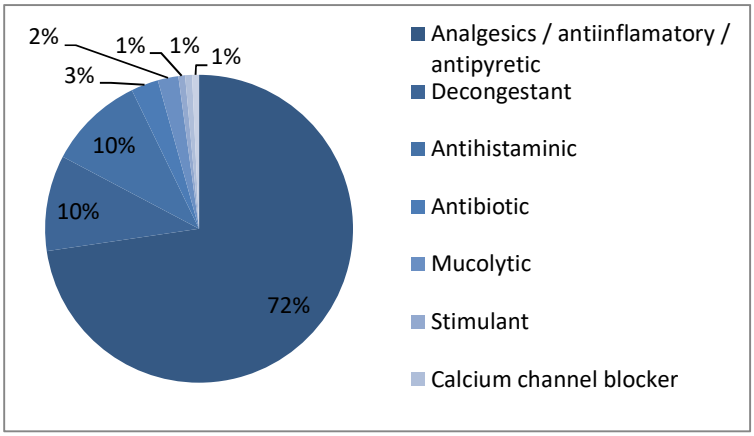


Figure 2.13. Distribution of over-the-counter medicines consumption by therapeutic use.

Section 5: Disposal of medicines

According to the information provided by the people, most of the households interviewed are use to disposing of the excess medicines into the normal waste bin (Figure 2.14). Other methods used for disposal of medicines are covering up with soil in the garden, disposal into the compost, or into the irrigation pond.

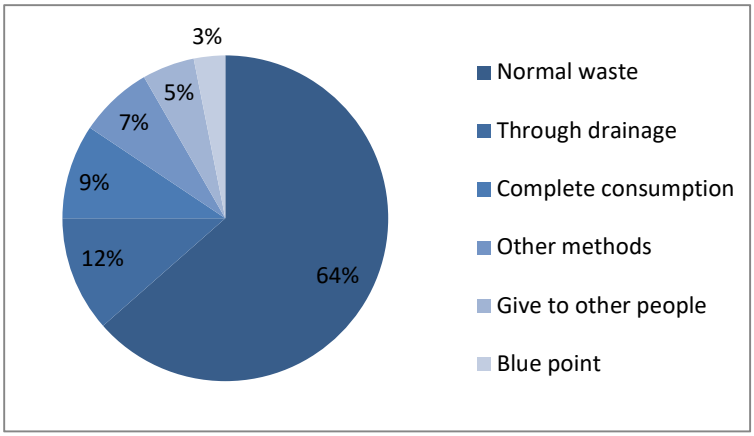


Figure 2.14. Methods used for disposal of medicines in surveyed households.

Section 6: Consumption of contraceptives

From 178 women, there were 100 between the reproductive ages of 15 – 50 years old. Among this group, 43% have contraceptive measures such as use of condom, pill, injection, intra uterine device (IUD), or subdermal implant. In Figure 2.15, the distribution of the female population as

interviewed regarding contraception methods is presented. In more detail, Figure 2.15 (right) shows the proportion of women that use the different contraceptive methods. In total, 55% of women are using hormonal contraceptives (pill, injection, subdermal implant). The hormones found in the contraceptive medicines are listed in Table 3.

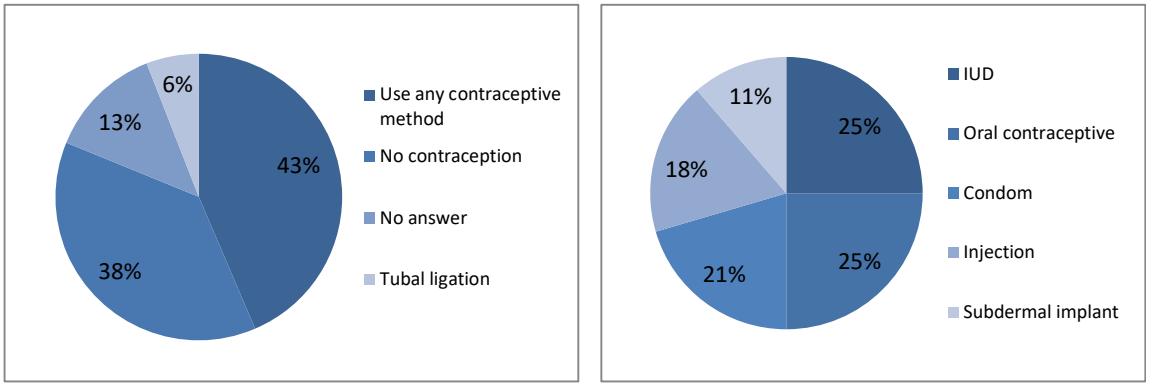


Figure 2.15. Use of contraceptive methods.

Left: Distribution of women in reproductive age (15 – 50 years old) by contraceptive measures. **Right:** Percentage of women that use any contraception method: barrier or hormonal contraceptives. IUD: Intra-uterine device.

Table 2.3. Most common synthetic hormones in contraceptive medicines consumed.

Hormonal contraception method	Active Pharmaceutical Ingredient
IUD	Levonorgestrel*
Oral contraceptives	Ethinylestradiol/ Levonorgestrel
	Drospirenone*
Injection / Oral	Medroxyprogesterone acetate*
	Norethisterone enanthate
	Estradiol valerate*
	Estradiol cypionate*
Subdermal implant	Etonogestrel
	Levonogestrel

*also used in hormone replacement therapy.

2.4 Discussion

This case study in Bogotá was performed with the purpose to understand the actual situation of sanitation and to identify the strengths and limitations of implemented ecological sanitation strategies. Also it was an opportunity to survey the consumption of pharmaceuticals that eventually are going to reach the environment.

Ecological sanitation and urine recycling in peri-urban and rural areas of Bogotá

Implementation of ecological sanitation was a measure to increase the safe access to water and sanitation mainly in the peri-urban and rural areas of Bogotá. However, the decline in the number of installed and active ASUs from 32 in 2008 to 6 in 2013 evidenced that somehow the initiative failed in maintaining the ASUs as well as in replicating them to other households.

Just after the project started, Montes (2009) evaluated the impacts and management of UDDTs in ASUs installed in households of rural areas of Suba. The author refers that the first year of implementation, monitoring was frequent and families were instructed on maintenance of toilets. Families were very enthusiastic with having the new facilities and were willing to change some cultural barriers. As proposed initially in the project, technical visits would be less frequent with the time, to the point families would take over the responsibility of management and maintenance. When that time came, bad odors and flies started to emerge in some households as they did not take proper care of UDDTs. Since most of the households kept their old flushing toilets many went back to the old habits.

This demonstrates the lack of capacity-building and support from implementing organizations, the weak experience on ecological sanitation in those organizations, and the deficient strategies of involvement of families in the project process and in the management of the sub-products. Many Ecosan projects have failed and some studies have proven that the two main reasons for not achieving sustainability in those projects are the technical problems and the lack of ownership which was reflected in the inadequate operation and maintenance (Rautanen and Viskari 2006).

Therefore, projects addressing ecological sanitation need to consider a holistic approach and need to be planned recognizing beneficiaries, their needs and local components (e.g, geographical, environmental, socio-economical, cultural, and political). Implementation must be conceived to be sustainable, with strong participatory methods to empower the society. The commitment of local people is one if not the most important pillar for the sustainability of Ecosan projects. Furthermore, monitoring should be continuous throughout the project to follow the progress' successes and failures and to make changes in the project in order to achieve the initial objectives. Last but not least, the project must be evaluated in order to analyze the achievement of objectives, the effectiveness of the project actions as well as the impacts and sustainability.

Although the Ecosan initiative in Bogotá was not highly successful, it was still important to observe that households with active ASUs were motivated, maintained all of the equipment that

was installed, a proper use of the UDDT, and a proper re-use of urine and feces. Furthermore, a family's economy that is based on self-subsistence agriculture has had a positive impact due to a lower investment on commercial chemical fertilizers. Another significant improvement is that infectious diseases have been less frequent since the equipment has been in use. These ASUs are evidence that the system can be sustainable and work at household level. Therefore, the Ecosan project initiative in Bogotá should be re-instituted.

Analysis of consumption of pharmaceuticals by survey

A survey of 96 households covered a wide range of age, socio-economical levels and education. However, this represents approximately 0.004% of the total population of Bogotá (~8 mi), a rather small sample size and not representative for the total population. For this reason, a descriptive analysis was used to summarize the gathered information. A bigger sample size would have given us a higher chance of collecting information from SEL 1, 5 and 6. Access to households of those SEL was more difficult due to security reasons (in the case of SEL 1), and to absence and limited willingness from households of SEL 5 and 6. In addition, the limited time to conduct the field activities was a constraint.

The survey was designed in a way such that as much detailed information as possible could be obtained from the household in less than 10 min. Some individuals were more reluctant to stay longer than 10 min answering questions since there were no immediate benefits to them. In general, the survey worked well for gathering the information required. However, for some questions (as in the case of illness treatment in Section 2), the interviewee could choose more than one answer option (question with more than one answer). Although it was possible to use the information, this made the data analysis and interpretation more challenging.

According to our results, a number of individuals over 60 years of age are prescribed daily doses of antihypertensives and analgesics/anti-inflammatories, which are the most frequently consumed pharmaceuticals. According to the report of Secretaría Distrital de Salud (2015), cardiovascular conditions represent the principal cause of mortality with arterial hypertension generally being the highest risk factor in Bogotá and Colombia. The survey also shows analgesics/anti-inflammatories are the most commonly used over-the-counter pharmaceuticals in Colombia and this is in accordance to other studies (López et al. 2009).

In a study on wastewaters and surface waters performed in Bogotá, Hernández et al. (2015) reported that compounds most frequently detected were the analgesics/anti-inflammatories acetaminophen, diclofenac, ibuprofen and lidocaine. The antibiotics clarithromycin and lincomycin, the antihypertensives valsartan, losartan, metoprolol and the antiepileptic

carbamazepine were also observed. Antihypertensives and analgesic/anti-inflammatory pharmaceutical compounds have been also frequently detected in wastewater treatment facilities in various European countries, Brazil and North America (Miège et al. 2009). Bayer et al. (2014) for example reported that antihypertensives of the sartan group (e.g., losartan, valsartan, irbesartan) were all detected in wastewater effluents and Bavarian rivers in southern Germany in higher orders of magnitude based on *µg* per liter and *ng* per liter, respectively. Ternes (1998) detected analgesics and anti-inflammatories such as diclofenac, ibuprofen, indometacine, naproxen, ketoprofen and phenazone in municipal sewage treatment discharges in Germany. Diclofenac showed the highest concentration in rivers and streams (0.15 µg L^{-1}). Acetylsalicylic acid was detected in 22 of 49 sewage treatment effluents and in rivers and streams, although the author suggests it is ultimately biodegradable.

The current results show that the disposal of expired or unused medicaments is done mostly through the trash and flushed down the household drain. Surveys in other countries, such as Germany, Austria and UK, show a similar pattern (Kümmerer 2009). Consequently, since 2011, the initiative "blue point" has been implemented in Colombia with the purpose of collecting unused and expired medicaments. The objectives of the blue point' containers are to avoid illegal manufacture of pharmaceuticals and inappropriate disposal. Unfortunately, people are not well informed about this option. The survey results showed that only one person was aware of the "blue points" and had actually used it.

Disposing of pharmaceutical products in the trash or down the drain means that those pollutants will eventually end up in the river streams. It is estimated that 96% of solid waste produced in Bogotá is dumped into the Doña Juana landfill. Actual leachate treatment in the landfill includes a physico-chemical and biological treatment to remove heavy metals, suspended solids, and reduce total nitrogen (ESMAP 2011). However, Hernández et al. (2015) observed that the surface waters of the Tunjuelo River, (the receiving stream of untreated sewage from ~2 mi people from south of Bogotá and the treated leachates from the Doña Juana landfill), showed similarities in the profile of pharmaceutical products to the wastewater collected in the influents of the El Salitre wastewater treatment plant. El Salitre is the only wastewater treatment plant in Bogotá and it treats the sewage of approximately 2.5 mi inhabitants (~25% of the total population). Its primary treatment is a chemically assisted process that removes 40% of organic matter and 60% of total suspended solids. The treated water is then discharged to the Bogotá River (CAR, 2017).

2.5 Conclusions

Although the implementation of an ecological sanitation approach in peri-urban and rural areas of Bogotá was not highly successful, the remaining ASU units showed the potential to improve sanitation, the safe management of household sewage, the safe access to drinking water, and the safe reuse of human feces and urine in agricultural fields. Therefore, more ecological sanitation projects need to be implemented with stronger planning, with implementation methodologies, with the empowering of involved communities, and with the monitoring and evaluation of the projects.

An assessment of the pharmaceuticals used and consumed by a larger population size in Bogotá would yield a better approximation to reality and also would facilitate the identification of the most consumed drugs (e.g., the anti hypertensives and analgesics/antiinflamatory) and the recalcitrant emerging pollutants in wastewater treatment plants and river streams. Furthermore, it would be important to correlate the pharmaceutical consumption and excretion rate in human urine and feces to assess their impact on the agricultural systems in other tropical scenarios.

Since there will always be a need for a cure or relief from many health conditions and diseases, the consumption of pharmaceuticals will not cease. Therefore, the pharmaceutical industry needs a more sustainable approach that will guaranty that pharmaceutical compounds are (bio)degradable in countries or regions lacking the sophisticated wastewater treatment processes. Additionally, the people should be introduced to good disposal practices of pharmaceutical products, especially in places such as Bogotá, which has such an ineffective management of wastewaters and solid waste.

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3 Yeast Estrogen Screen assay for measuring estrogenicity in soil, plant tissues and diluted human urine

3.1 Introduction

Chemical and biological techniques have been used to monitor estrogenic compounds and estrogenic potencies in environmental samples. Whereas chemical methods identify target compounds and quantify their concentration within the sample, the biological techniques determine the estrogenic activity exhibited by a chemical or group of chemicals in a sample (Gómes et al. 2003). Among the chemical techniques available for the analysis of estrogenic compounds, liquid chromatography (LC) and gas chromatography (GC) coupled with mass spectrometry (MS) are the most widespread (Gabet et al. 2007, Streck 2009). Both techniques have been used to analyze surface waters and wastewaters (e.g., Baronti et al. 2000; Belfroid et al. 1999; D'Ascenzo et al. 2003; Yost et al. 2013; Zheng et al. 2008), and soils and river sediments (Beck et al. 2008; Goeppert et al. 2014; Hájková et al. 2007). High performance liquid chromatography (HPLC) has been frequently used for analysis of soils and river sediments as well (Colucci and Topp 2002; Das et al. 2004; Karnjanapiboonwong et al. 2010; Lee et al. 2003; Lucas and Jones 2006; Mashtare et al. 2002; Scherr et al. 2009; Sun et al. 2010; Van Emmerik et al. 2003). Analyses of phytoestrogens (e.g., lignans, isoflavones) in plant samples have been carried out mostly by GC-MS (Čukelj et al. 2011; Liggins et al. 2000; Peñalvo et al. 2005; Thompson et al. 2009), and HPLC (Smeds et al. 2009).

Biological techniques include competitive ligand binding assays, cell proliferation assays and recombinant receptor-reporter assays. Those techniques have been already described in detail by, amongst others, Streck (2009) and Zacharewski (1997). The detection of estrogenicity in environmental samples has been performed, especially, by recombinant receptor-reporter assays, from which the YES (Yeast Estrogen Screen) assay has been developed. Yeast Estrogen Screen measures the activation of the receptor, as this quantifies the estrogenic potency of a substance. The YES assay has gained more popularity since it is simple to handle, inexpensive, and possess high sensitivity to the level of *ng per Liter*. Campbell et al. (2006) suggested that yeast-based assays have the advantage of being less susceptible to non-sterile conditions as compared to those based on mammalian or fish cell lines. Therefore, YES assay is more suitable for complex environmental samples (e.g., sewage sludge). Furthermore, yeast does not possess hormonal receptors endogenously which is advantageous if endocrine-disrupting compounds are to be detected. Different studies have successfully used YES assay alone or combined with

chemical approaches, primarily for monitoring estrogenicity in surface waters and wastewater treatment effluents (e.g., Aerni et al. 2004; Beck et al. 2006; Nelson et al. 2007; Pawlowski et al. 2004; Rutishauser et al. 2004; Salste et al. 2007; Takigami et al. 2000). Up to now, the monitoring of estrogenicity by means of YES assay in soils and plant material has not been sufficiently documented.

The YES assay allows for the screening of a high number of samples, which is more cost effective, and it requires a low investment in facilities and equipment as compared to other analytical methods. This is beneficial, especially for developing regions, such as Colombia, that generally have a low-budget for studies, but an increasing interest in monitoring such micropollutants in the environment.

Given the advantages of using the YES assay, this study intends to test its potential use for screening estrogenicity in a soil-plant culture system, as well as in diluted human urine (DU). For this purpose, a type of soil, a type of sediment, dried spinach leaves, milled wheat grains from the market, and DU from a private household as well as an office building (GIZ headquarters) were tested.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Chemie (Germany). MeOH (99.9% purity) HPLC Ultra Gradient Grade (UGG) ROTISOLV®, acetone (99.9% purity) HPLC UGG ROTISOLV® and water HPLC UGG ROTISOLV® were purchased from ROTH (Germany). Ultra pure water was obtained from a Millipore Quantum®Ex purification system.

3.2.2 Recombinant Yeast Estrogen Screen assay (YES)

The recombinant YES assay and the proceedings for preparation of the medium have been described in detail by Routlege and Sumpter (1996). A recombinant yeast strain (*Saccharomyces cerevisiae*) was obtained from Dr. Sumpter of Brunel University (Middlesex, UK). The DNA sequence of the human estrogen receptor (hER) has been integrated into the yeast cells genome. The yeast strain also contains the expression plasmids carrying estrogen responsive elements regulating the expression of the reporter gene Lac-Z, encoding the enzyme β-galactosidase. When the cells are incubated in the presence of estrogenic compounds, the reporter gene Lac-Z produces β-galactosidase into the medium and causes the chromogenic substrate, chlorophenol red-β-D-galactopyranoside (CPRG), to undergo a color change from yellow to red. This color change can be quantified by measuring the absorbance at a wave length of 540 nm. For the

purpose of this study and following the recommendations of Le (2012), some modifications to the method proposed by Routlege and Sumpter (1996) have been made. To avoid bacterial contamination on the assay plates, 100 µL of an antibiotic mixture (8 mg penicillin and 8 mg streptomycin in 10 mL sterilized doubled distilled water) was added to 50 mL of growth medium.

3.2.3 Protocol for the detection of estrogenic active compounds

All material and surfaces must be properly clean and sterilized. Contamination with estrogenic chemicals will lead to an elevated background expression. All YES assay procedures should be done under an air flow cabinet.

Preparation of medium components

Minimal medium (pH 7.1; 1 L HPLC UGG water)

KH_2PO_4	13.61 g
$(NH_4)_2S0_4$	1.98 g
KOH pellets	4.2 g
$MgSO_4$	0.2 g [0.41 g $MgSO_4 \cdot 7H_2O$ yields 0.2 g $MgSO_4$]
$Fe_2(S0_4)_3$	1 mL solution [40 mg per 50 mL H_20]
L-Ieucine	50 mg
L-histidine	50 mg
Adenine	50 mg
L-arginine-HCI	20 mg
L-methionine	20 mg
L-tyrosine	30 mg
L-isoleucine	30 mg
L-lysine-HCI	30 mg
L-phenylalanine	25 mg
L-glutamic acid	100 mg
L-valine	150 mg
L-serine	375 mg

The solution must be placed on a heated stirrer to dissolve the solids. 45 mL aliquots are dispensed into 250 mL flasks, and later sterilized at 121°C for 10 min. Store at room temperature.

D-(+)-Glucose solution – Total volume: 200 mL

Prepare a 20% w/v solution. Use HPLC UGG water. Dispense two 100 mL aliquots in glass bottles, sterilize at 121°C for 10 min. and store at room temperature.

L-Aspartic acid solution

Prepare a stock solution of 4 mg per 1 mL HPLC UGG water. Dispense 10 mL aliquots in brown glass vials, sterilize at 121°C for 10 min. and store at room temperature.

Vitamin Solution – Total volume: 180 mL

Thiamine	8 mg
Pyridoxine	8 mg
Pantothenic acid	8 mg
Inositol	40 mg
Biotin solution	20 mL (2 mg per 100 mL HPLC UGG water)

Sterilize the solution by filtering through a 0.2 μm pore size Watman PURADISC disposable filter (or similar), in a laminar air flow cabinet. Dispense 10 mL aliquots into sterile brown glass vials and store at 4 °C.

L-Threonine – Total volume: 100 ml

Prepare a solution of 24 mg per 1 mL HPLC UGG water. Dispense 10 mL aliquots into sterile brown glass vials, sterilize at 121°C for 10 min. and store at 4 °C.

Copper (II) Sulfate – Total volume: 50 ml

Prepare a solution of 20 mM (159.6 mg per 50 mL HPLC UGG water) and sterilize by filtering through a 0.2 μm pore size Watman PURADISC disposable filter (or similar) in a laminar flow cabinet. Dispense 5 mL aliquots into sterile brown glass vials and store at room temperature.

Preparation of growth medium

Glucose solution	5 mL
L-aspartic acid solution	1.25 mL
Vitamin solution	0.5 mL
L-threonine solution	0.4 mL
Copper (II) sulfate solution	125 μL

The above solutions are added to a 45 mL previously prepared minimal medium. Inoculate the growth medium with 125 µL of concentrated yeast stock and incubate at 28°C on an orbital shaker (~200 rpm) for approximately 24 hours or until it reaches an absorbance of 0.9-1.0 at a wave length of 630 nm. The absorbance indicates the appropriate yeast cells biomass.

Chlorophenol red-β-D-galactopyranoside (CPRG)

Prepare a 10 mg per 1 mL HPLC UGG stock solution and sterilize by filtering through a 0.2 µm pore size filter. Dispense 1 mL aliquots into sterile cryogenic vials in a laminar flow cabinet and store at -20°C.

3.2.4 YES assay procedure

In this study the growth medium (50 mL) was seeded with 2 mL yeast from a 24h culture and 0.5 mL of CPRG solution was prepared. Each microtitre plate consisted of a row of blanks (100% MeOH), a row of standards of βE2 (concentrations from 2 to 2700 ng L^{-1}) and 13 samples. Four replicates of 10 µL aliquots of blanks, standards and samples were transferred to the wells of a sterilized 96-well optical flat bottom microtitre plate (Nunc, Germany). MeOH was allowed to evaporate until dryness and 200 µL of the growth medium with CPRG was given using an 8-channel pipette (Transferpette®-8 50-200 µL BRAND, Germany). Plates were incubated at 32°C over 3 d. Every day, plates were shaken for 3 min. After 3 d. of incubation, absorbance was read using BIOTEK ELx 800™ microplate reader and the analysis software Gen5™. The color development was measured at a wavelength of 540 nm and the turbidity of yeast cell biomass was read at 630 nm. The turbidity values were adjusted using the equation:

Corrected value = Test absorbance 540 nm – (Test absorbance 630 nm – Blank absorbance 630 nm)

3.2.5 Extraction of estrogenic compounds

Sample preparation

Soils: two different types of substrate were used: Meckenheimer topsoil (MTS) (Luvisol derived from loess) and a standard pot substrate mix (SPM) (70 % white peat, 20 % loam, 10 % perlite. Gepac, Type VM, Sinntal-Jossa, Germany). A more detailed description of the MTS can be seen in Table 3 of Chapter 5. Substrates MTS and SPM were oven dried for 72 h at 60°C and finely milled using a SIEBTECHNIK® disc mill, to pass a 300 µm sieve.

Plant tissues: spinach leaves (SL) and packed wheat grains (WG) bought in the supermarket were used. Spinach leaves were prepared as described above for soils. WG was directly milled.

Diluted human urine (DU) refers to human urine diluted with flushing water. DU-GIZ was collected from the main building of GIZ (Deutsche *Gesellschaft für Internationale Zusammenarbeit* GmbH- Eschborn, Germany) where 23 waterless urinals (Centaurus KERAMAG, Germany) and 38 urine-diversion flush toilets (Roediger, Germany) have been installed. From there, DU is sent to the 2.5 m^3 collection tanks. Approximately, 40 m^3 of DU per year are collected in this facility. Also, DU-PH was obtained from a private household where a Gustavsberg Separation Toilet (Berger Biotechnik® GmbH) has been installed. Dilution factor of DU-PH can be seen in chapter 4, section 4.2.1 and physico-chemical properties can be seen in chapter 5, Table 5.3. For the purpose of this study, 1 m^3 DU was obtained from both DU-GIZ and DU-PH. Samples were collected from the 1 m^3 tanks and were combined into a composite sample as follows: four samples of 250 mL each were collected and thoroughly mixed. From this composite sample, four samples of 10 mL each were extracted and analyzed with YES assay.

Extraction of solid samples

Basically, extraction procedure is identical for solid samples. However, some modifications occurred in the extraction of spinach leaves and wheat grains which are explained below.

1. Eight replicates of 5g (dry weight) milled samples (MTS, SPM, SL) were weighed into 50 mL Erlenmeyer flasks.
2. Four replicates were spiked with 20 µg L^{-1} 17β-estradiol (βE2) and the other four did not receive any βE2.
3. **First round of extraction:** All flasks received 7 mL of MeOH 100% and were left to rest for 30 min. Flasks were covered with aluminum foil.
4. Flasks were shaken in a water-bath orbital shaker (Grant OLS 200) for 30 min, at 40°C and 150 rpm.
5. Flasks were then left to rest for 30 min at room temperature.
6. The supernatant was collected into 50 mL sterile polypropylene centrifugation tubes (Eppendorf).
7. **Second round of extraction:** steps 3 to 6 were repeated.
8. The supernatant was centrifuged (Eppendorf Centrifuge 5810R) for 10 min, at 4°C and 4000 rpm.
9. 10 mL supernatant were transferred to a 20 mL glass vial.
10. Glass vials were placed on a heater block (Stuart SB-H130D/3) at 40°C and allowed the liquid to evaporate under nitrogen gas (Techne sample concentrator).
11. Evaporated samples were eluted with 500 µL MeOH 100% into 1.5 mL amber glass vials and kept at -20°C until the sample analysis.

Spinach leaves (SL)

SL test 1: spiked and non-spiked samples received 20 mL of MeOH 100% and 10 mL MeOH 100% for the first and second round of extraction, respectively (Step 3 and 7). The following steps continued as described above for extraction of solid samples.

SL test 2: spiked and non-spiked samples received 20 mL of MeOH 100% and 10 mL MeOH 100% for the first and second round of extraction, respectively (Step 3 and 7). For this group of samples, shaking in a water-bath was replaced by ultrasonication (Step 4). An ultrasonic cleaning bath model Bandelin SONOREX SUPER RK 102H, with an ultrasonic frequency of 35 khz was used. The bath was kept at a constant temperature of 30°C. Samples were ultrasonicated for 30 min.

Wheat grains (WG)

10 g of material were used per replicate.

WG test 1: spiked and non-spiked replicates received 15 mL of MeOH 100% and 10 mL MeOH 100% for the first and second round of extraction, respectively (Step 3 and 7). The following steps continued as described above for extraction of solid samples.

WG test 2: spiked and non-spiked replicates received 15 mL of MeOH 100% and 10 mL MeOH 100% for the first and second round of extraction, respectively. Samples were ultrasonicated for 30 min as explained above for SL test 2.

To calculate the recovery (%) of βE2 in each sample, the extract was analyzed by YES assay, and the βE2 equivalent value (βE2 EQs) in the non-spiked sample was subtracted from that in the spiked sample.

Extraction of diluted human urine (DU)

Estrogenic compounds in DU were extracted by Solid Phase Extraction using Strata™-X polymeric SPE columns (8B-S100FCH) 200 mg/6 mL, connected to a Tall Boy™ - 10 position vacuum manifold (Phenomenex). SPE columns were activated with 7 mL of MeOH 100% and then washed with 7 mL of MeOH 5%. After this, 10 mL DU was loaded onto the SPE column and drained with a flow rate of 5.5 – 6 mL/min. Before the column was empty, 7 mL of MeOH 50% was loaded, followed by 7 mL of acetone:water (1:2). SPE columns were dried under nitrogen gas (99.9%) for at least 30 min. After this, the compound was eluted with 7 mL of MeOH 100% into sterile glass vials and kept at -20°C until analysis.

3.3 Results

3.3.1 Extraction efficiencies of βE2 in soils and plant material

Values of recovery percentage of 17β-estradiol spiked in the different tested samples are shown in Table 3.1. The results suggested that the extraction method was successful for soils and no modifications in the procedure were necessary. Recovery % of both MTS and SPM was more than 100%. The concentration of non-spiked soil samples was found to be 0.05±0.01 μg kg^{-1} βE2 EQs and 0.06±0.02 μg kg^{-1} βE2 EQs in MTS and SPM, respectively.

Table 3.1.Recovery % of 17β-estradiol spiked and its standard deviation.

Material	Recovery % ± STDEV %
Soil	
Meckenheimer top soil (MTS)	107.6±19.5
Standard pot substrate mix (SPM)	104.3±20.1
Spinach (SL)	
SL test 1 (Water-bath agitation)	23.9±15.2
SL test 2 (Ultrasonication)	1.9±7.9
Wheat grains (WG)	
WG Test 1 (Water-bath agitation)	66.1±19.8
WG Test 2 (Ultrasonication)	96.2±14.3

In WG test 2, treated with ultrasonication, the extraction recovery was higher as compared to WG test 1. Concentration in non-spiked samples of wheat grains was 0.25±0.29 μg kg^{-1} βE2 EQs.

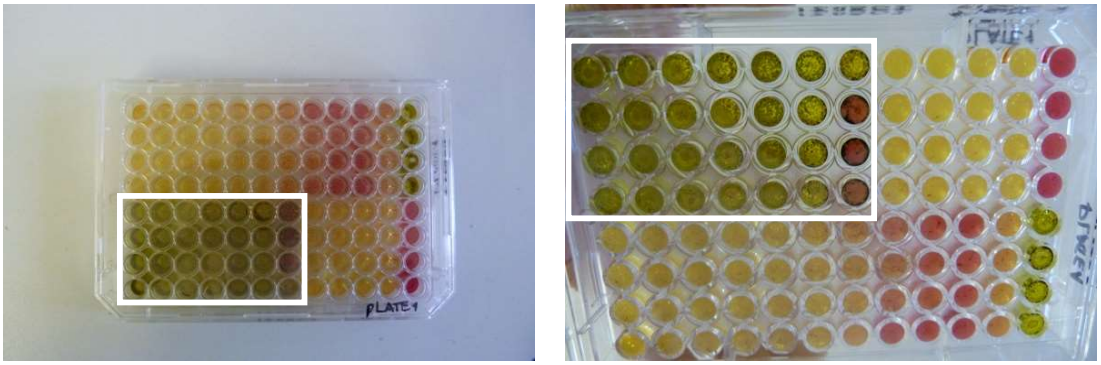

Figure 3.1.Top view onto a microtitre plate.

Left: White square shows the wells with SL samples. **Right:** Detailed view of wells with SL samples. Sediments accumulated at the bottom can be observed.

In both extraction tests with spinach (SL Extraction test 1 and 2), the recovery was low. However, SL test 1 showed an increased recovery %. In samples exposed to ultrasonication (SL test 2), it was observed that a high content of wax and fat remained after evaporation and

elution of samples was much more difficult. Additionally, in both SL test 1 and SL test 2, a high amount of sediment was accumulated at the bottom of the wells during the incubation period (Figure 3.1). Consequently, it resulted in false readings.

3.3.2 Estrogenic potency in DU

Table 3.2 shows the results of YES screening of DU. DU-GIZ has a higher estrogenicity (βE2 EQs) than DU-PH.

Table 3.2. Estrogenic potency in DU from private household and GIZ office building

Diluted urine estrogenic potency	$\mu g\ L^{-1}$ βE2 EQs ± STDEV
DU- PH	0.42±0.07
DU- GIZ	2.46±0.19

3.4 Discussion

This study showed that the extraction process of estrogenic compounds used for both soil types (MTS and SPM) and wheat grains (WG) was appropriate for screening estrogenicity with the YES assay. The extraction protocol used in this study was based on the procedure used by Le (2012), which confirmed that the extraction process and the use of YES assay is appropriate for monitoring estrogenic compounds in environmental samples such as, surface river water and river sediments, black water from septic tanks, cow and pig manure, and wastewater.

Still, studies using YES assay for monitoring estrogenicity in soils of agricultural environment and other sediments are very scarce. Langdon et al. (2014) evaluated the persistence of estrogenic activity in soils after application of biosolids. These authors suggested that assay was useful to verify that the estrogenicity persisted for at least 4 months after biosolids field application. They also indicated that the method is very reliable, though it does not identify target compounds. Viganò et al (2008) compared the potential of an immunoassay (ELISA), a bioassay (YES) and a chemical approach (LC-MS/MS) for analysis of river sediments in Italy. The authors suggested that results obtained with YES demonstrated to be closer to LC-MS/MS, while ELISA tended to overestimate the relative concentration of estrogens, and it was highly susceptible to interferences from other chemicals.

To our knowledge, there have been no studies using YES assay for analysis of estrogenicity in plant tissues. Thus, different extraction procedures were studied. As in case of wheat grains, the recovery % of βE2 was higher after ultrasonication. Toma et al. (2001) confirmed that ultrasonication boost the penetration of solvent into the dried plant material and this wetting process may enhance the extraction of compounds, but Soares Melecchi et al. (2006) indicated

that the most influential factors for ultrasonication assisted extraction are solvent polarity and extraction time. The authors also suggest methanol as a suitable solvent for a broad range of compounds and an approximate time of 140 min as optimal for an efficient extraction. In this study, the use of methanol as extraction solvent for βE2 gave positive results. Furthermore, the extraction of estrogens in wheat grains did not require more than 30 min of exposure to ultrasonication. The present results also suggest that ultrasonication may enhance the extraction of lignans or phytoestrogens contained in wheat grains, which explains the high recovery rates.

The ultrasonication of spinach leaves resulted in a high yield of lipid and wax components. Toma et al. (2001) suggested that a lower ultrasonic vibration frequency (20 kHz) has a higher effect on swelling and extraction of oils and resins than a high vibration frequency (500 kHz). The ultrasonic vibration used in our extraction procedure was in the low range (35 kHz). Adjustment of the vibration in our equipment was not possible; therefore this needs to be considered for future tests. It is believed βE2 could have bound to this lipidic phase due to its lipophilic nature, which explains the low recovery.

High turbidity and chlorophyll contents in the SL samples raised additional problems for exact analysis. For that reason, a cleaning step using SPE columns (as explained in section 2.3.2) was tried, but neither chlorophyll, nor turbidity were totally eliminated. It is highly probable that the chlorophyll and/or the sediments accumulated at the bottom of the wells interfered with the correct absorbance measurement resulting in non-uniform data and a high standard deviation.

Up to now, studies related to the use of YES assay for analysis of plant material are very scarce, except for a study by Takamura-Enya et al. (2003). The authors aimed to determine estrogenicity in different food items including different vegetables (e.g., beans, carrots, horseradish, sesame and spinach). However, the extraction method used for vegetables is not described in detail, which makes a comparison of the methods difficult if not impossible. The authors do not report any estrogenic potency values for the vegetables analyzed.

This study proved that the extraction protocol for river water proposed by Le (2012) can be also used for extraction of DU. Screening of estrogenic potency in DU with YES assay evidenced the difference between DU-GIZ and DU-PH. As expected, DU-GIZ showed a higher estrogenic potency than DU-PH, which can be explained by the higher number of donors and a more diverse population group. Presence of women under contraceptive treatment, hormonal treatment, and/or pregnancy is highly probable. Also, the storage time in GIZ facilities was longer than six months, which may also give opportunity for deconjugation of estrogens excreted as conjugated

forms (See chapter 4). In contrast to DU-GIZ, DU-PH comes from a male and a female couple that do not consume any additional hormones.

Takigami et al. (2000) used the YES assay for the analysis of raw night soil, a mixture of human urine and feces. The estrogenic potency they found (~8 μg L^{-1} βE2 EQs) was approximately 4 times higher than the one analyzed for DU-GIZ in this study. The presence of feces may increase significantly the estrogenicity in a sample since it contains mainly free estrogens that have undergone deconjugation by enteric bacteria. In urine, estrogens are mainly excreted in conjugated forms, which means inactive and thus its estrogenic activity cannot be detected by YES assay. Activation of conjugated forms is mainly performed through bacteria (Khanal et al. 2006).

3.5 Conclusions

It has been confirmed that the extraction protocol proposed in this study can be used for soil, wheat grains and DU. Furthermore, YES assay appears to be a good tool for screening estrogenic potency in soil, wheat grains and DU.

Unfortunately, extraction of spinach samples presented difficulties resulting in erroneous results when measuring estrogenic potency. Therefore, it is recommended testing new extraction methods for plant material. Experiments with other plant species may help to avoid the difficulties encountered in this study. Ultrasonication might be an important step for extraction procedure; however it must be carefully used to minimize extraction of undesired substances in the matrix (e.g. oils and resins).

3.6 References

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4 Influence of storage conditions on the estrogenic activity in diluted human urine

4.1 Introduction

Use of human urine as fertilizer is an ancient practice that lately has gained more interest, especially as a nutrient source in developing and transition countries. Human urine contains an important amount of nutrients readily available for plants that makes it as efficient as mineral fertilizers (Akpan et al. 2012; Heinonen-Tanski et al. 2007; Germer et al. 2011; Guzha et al. 2005; Pradhan et al. 2009; Shrestha et al. 2013). Besides, recycling of urine reduces fertilizer cost investment and pollution effects from unsafe excreta disposal plus costly wastewater treatments.

Despite the benefits, reutilization of human excreta in agriculture has been associated to health and environmental risks and socio-cultural issues (WHO 2006). Urine is a waste product that concentrates the metabolites and noxious substances the human body has to dispose of. Therefore a range of pollutants, such as residues of pharmaceutical compounds and natural and synthetic hormones, can be found in urine (Lienert et al. 2007; Curry and Whelpton 2017). It has been suggested that micropollutants could accumulate in soils and might be taken up by plants through the use of untreated urine as fertilizer and thus enter the food chains (Winker 2009).

Specifically, accumulation of natural and synthetic estrogenic compounds in the environment has received considerable attention as they act as endocrine disruptors (Khanal et al. 2006). Different effects on aquatic organisms have been observed such as alteration in development of genitals, presence of hermaphrodites, changed mating behavior etc. (Hamid and Eskicioglu 2012). Despite the undeniable evidence, health effects on humans are still not clearly confirmed, but if estrogenic disruption occurs in aquatic fauna, most probably it will also affect humans and other vertebrates (Welshons et al. 2003).

Although human urine contains as many as 15 natural estrogens (Xu et al. 2005), steroidal estrogens estrone (E1), 17β-estradiol (βE2) and estriol (E3) and their respective conjugates are the most abundant. It has been reported that premenopausal women can excrete an average rate of 7.3-13.8 μg day^{-1} of E1, 2.8-3.6 μg d^{-1} of E2 and 4.5-13.6 μg d^{-1} of E3 (Liu et al. 2009), while men can excrete rates up to 4 μg d^{-1} E1, 1.5 μg d^{-1} E2 and 1.5 μg d^{-1} E3 (Fotsis and Adlercreutz 1987). Together with the synthetic estrogen 17α-ethinylestradiol (EE2), steroidal estrogens E1,

βE2 and E3 are most common in wastewater and apparently the main endocrine disruptors in the environment (Racz and Goel 2010).

Different strategies for removal of estrogens from water bodies and wastewater have been studied (Silva et al. 2012). Estrogens E1, βE2 and the synthetic EE2 are subject to direct and indirect photodegradation in water bodies under natural and simulated sunlight conditions (Atkinson et al. 2011; Caupos et al. 2011; Chouwdhury et al. 2010; 2011; Leech et al. 2009; Liu et al. 2003a,b; Liu and Liu 2004; Mazellier et al. 2008; Zhang et al. 2007). The same compounds can be as well degraded by microorganisms, though various studies indicate that estrogens are not easily broken down as residues are found in sewage plant effluents and surface water bodies (Baronti et al. 2000; Belfroid et al. 1999; Ternes et al. 1999).

As there is a risk of spreading estrogenic compounds onto the farming fields by urine or diluted urine application as fertilizer, it is important to understand the turnover of estrogens during its storage and hopefully reduce its activity. The present study thus aims at evaluating the potential degradation of naturally excreted estrogens in diluted urine (urine + flushed water) under simulated light and dark conditions, and to elucidate the influence of intrinsic parameters of diluted human urine (e.g. dissolved organic carbon, ammonium, pH, and electric conductivity) on the degradation process. We thus hypothesize that estrogenicity in human urine can be reduced by irradiation as well as by microbial activity in the dark.

4.2 Materials and methods

4.2.1 Source-separated diluted urine

Diluted urine was collected from a private household, where a Gustavsberg Separation Toilet (Berger Biotechnik® GmbH) has been installed. This toilet separates urine from solid waste by two different outlets. Flushing water volume is 2 L and 4 L for urine and solid waste, respectively. It is suggested by the manufacturer that ~10% of the flushing water used for solid waste reaches the urine siphon. Thus the urine obtained is diluted approximately by a factor of 1:8. For this reason, we refer to this fraction as diluted urine (DU). The donors, a male and female couple, had no further intake of pharmaceuticals or additional estrogens. For the purpose of this experiment, DU was not older than two weeks and not spiked with additional estrogens.

4.2.2 Experimental set up

The experiment consisted of two treatments: (1) DU under dark conditions and (2) DU exposed to light. Each treatment was repeated six times. For this purpose, 300 mL Erlenmeyer soda lime glass flasks were used. To create dark conditions, flasks were completely wrapped with

aluminum foil. In both treatments, 300 mL of DU per flask were used. Light exposed flasks were covered with crystal clear polystyrene lids, which allowed a light transmission of 90% of the 320-800 nm spectrum (BASF 2010). Before starting the experiment, all glassware was thoroughly washed, cleaned twice with absolute ethanol and kept in the oven at 180°C for four hours for surface sterilization. The same process was followed for cleaning the glass pipettes after sample collection. The experiment was set up in an environmental chamber PERCIVAL E-36HO equipped with compact fluorescent lamps (55W) and CLEO compact PLL lamps (36W) covering the range of UV-B, UV-A and visible light (280-700 nm). The environmental chamber was programmed to run with 10 h of darkness and 14 h of light. Relative humidity (RH=60%) and air temperature (23°C) were kept constant during day and night. Samples were collected at day 0, 1, 2, 3, 5, 7, 9, 12, 15, 20, 40, and 75, at a volume of two times 10 mL of DU from each of the six replicates and transferred into glass vials. One of the 10 mL replicate of every repetition was used to measure pH and electrical conductivity (EC) immediately after sample collection, and the other replicates were kept at -20°C until analysis. By using separate samples, contamination was avoided through pH and EC electrodes.

4.2.3 Chemicals

Chlorophenol red-β-D-galactopyranoside (CPRG) and 17β-estradiol were purchased from Sigma-Aldrich Chemie (Germany). MeOH (99.9% purity) HPLC Ultra Gradient Grade (UGG) ROTISOLV®, acetone (99.9% purity) HPLC UGG ROTISOLV® and water HPLC UGG ROTISOLV® were purchased from ROTH (Germany). Ultra pure water was obtained from a Millipore Quantum®Ex purification system.

4.2.4 Extraction of DU samples for analysis of estrogens

Estrogens in DU were extracted by means of Solid Phase Extraction (SPE) using Phenomenex Strata™- X polymeric reversed phase columns 200 mg/6 mL(8B-S100FCH), connected to a Tall Boy™ - 10 position Vacuum manifold (Phenomenex). Glassware was previously washed with distilled water, rinsed twice with absolute ethanol and sterilized in the oven at 180°C over night. SPE columns were activated with 7 mL of MeOH 100% and then washed with 7 mL of MeOH 5%. Thereafter, 8 mL DU was loaded onto the SPE column and drained with a flow rate of 5.5 – 6 mL min^{-1}. Before the column ran dry, 7 mL of MeOH 50% was loaded followed by 7 mL of acetone:water 1:2. SPE columns were dried under nitrogen gas (99.9%) for at least 30 min. Estrogens in DU were eluted from the dried columns by 7 mL of MeOH 100% into sterilized glass vials and kept at -20°C until analysis.

4.2.5 Analytical methods

Yeast Estrogen Screen (YES) assay

Estrogenic activity in DU was determined by the YES assay as described in Chapter 3. The calibration of the 17β-estradiol standard curve was performed with the 4 parameter logistic model (Findlay and Dillard 2007). Estrogenic activity is presented as concentration of 17β-estradiol equivalents (μg L^{-1} E2 EQs). The limit of detection (LOD) was calculated based on the volume of the sample used for extraction (8 mL), the volume of the eluted sample (7 mL) and the lowest concentration that can be detected in the assay plate (20 ng L^{-1}E2 EQs). In the present study, the LOD for DU samples amounted to 0.02 μg L^{-1} E2 EQs.

The concentrations of total nitrogen (N_{tot}), ammonium-N (NH_4^+-N) and dissolved organic carbon (DOC) were measured by means of a continuous flow analyzer (AutoAnalyzer3 BRAN+LUEBBE Germany). EC and pH were determined by a pH SenTix41 WTW electrode and the TetraCon® 325 EC electrode run with a Multi 340i WTW set.

4.2.6 Statistical analysis

Changes of estrogenic activity in diluted urine under light and dark conditions were calculated based on the assumption that natural estrogens E1, βE2 and E3 follow first order kinetics (Chowdhury et al. 2011). The pseudo-first order degradation rate constant was calculated from the slope of the logarithmic plot of estrogenic concentration as a function of time using equation 1, and the half-life of estrogenicity was calculated by equation 2

Equation 1 $\ln (C_{\beta E2EQs} / C_{0\ \beta E2EQs}) = -kt$

Equation 2 $t_{1/2} = \ln 2 / k$

where $C_{0\ \beta E2\ EQs}$ and $C_{\beta E2\ EQs}$ are the concentrations of estrogens at time zero and thereafter, k is the reaction rate (d^{-1}) and $t_{1/2}$ or half-life refers to the time in d needed to degrade 50% of the initial concentration.

Decay of estrogenic activity was calculated until day 20. Data corresponding to day 40 and 75 are shown but not used in the statistical analysis. Datasets were tested for homogeneity of variance and normal distribution (Shapiro-Wilk test). Additionally, a Pearson Moment Correlation Coefficient was obtained to understand the dependence between the variables evaluated. Statistical analysis was performed by using SigmaPlot 11.0.

4.3 Results

4.3.1 Estrogenic activity in DU under light and dark conditions

The average concentration of estrogenic activity in DU at day 0 was determined as 2.41 ± 0.7 µg L^{-1} βE2 EQs. The plot of $ln(C_{\beta E2\ EQs} / C_{0\ \beta E2\ EQs})$ versus time (Figure 4.1) indicated that after 20 d of incubation the decay of estrogenic activity was exponential and followed first-order-kinetics in both DU under light and dark conditions. The reaction rate (k) suggested that decay until day 20 was faster in DU under light (0.07 d^{-1}) than in dark (0.05 d^{-1}). Additionally, half life ($t_{1/2}$) was calculated as 10.05 and 12.60 d for DU under light and dark, respectively. After 20 d, 76% and 54% of initial estrogenic activity was lost in DU under light and under dark, respectively.

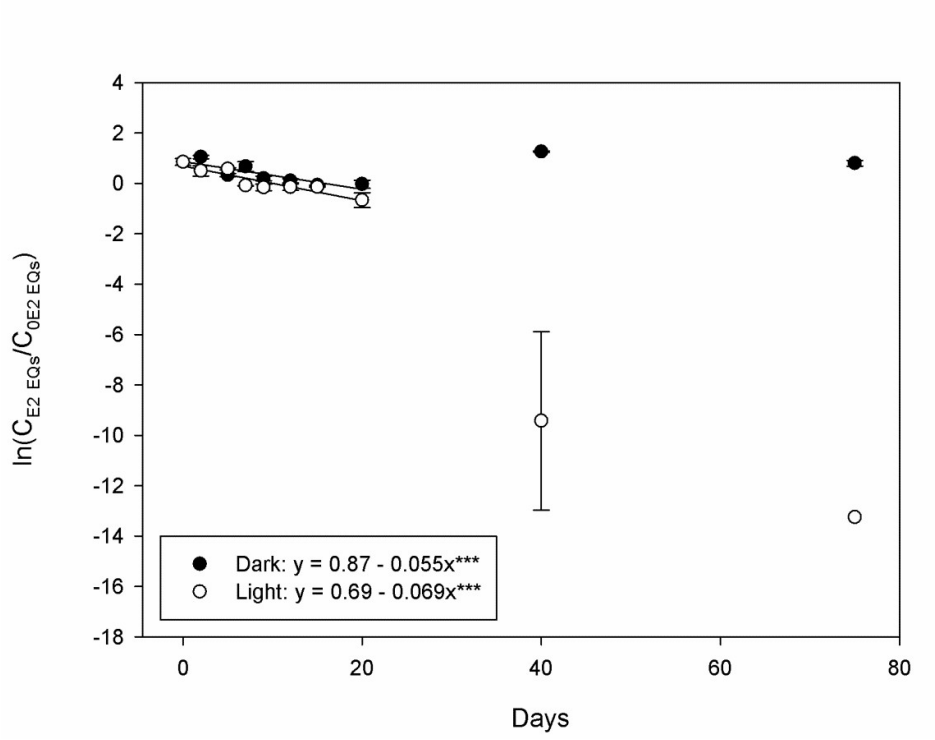

Figure 4.1. Decay rate of estrogenic activity in DU under dark and light conditions over time.
n= 4. *** p<0.0001. ● represents urine under dark; ○represents urine exposed to light. Error bars show standard deviation of the mean.

4.3.2 Dissolved organic carbon (DOC)

The average content of DOC in DU at day 0 was found as high as 986 mg L^{-1}. Along the experimental period, DOC decayed exponentially following the first-order-kinetics model (Figure 4.2). The reaction rate (k) indicated that DOC decay was higher in DU under dark (0.03 d^{-1}) than in DU exposed to light (0.02 d^{-1}). In both treatments, around 60% of the DOC decayed

before day 20. However, in DU under light, the degradation process slowed down between day 20 and day 75, while it seemed to continue in DU under dark conditions. Thus, DOC losses were 85% under dark and 72% under light conditions.

Within 20 days of incubation, estrogenic activity was significantly positive correlated to DOC in DU in dark ($r=0.721$; $p<0.001$; $n=32$) and light ($r=0.689$; $p<0.001$; $n=32$).

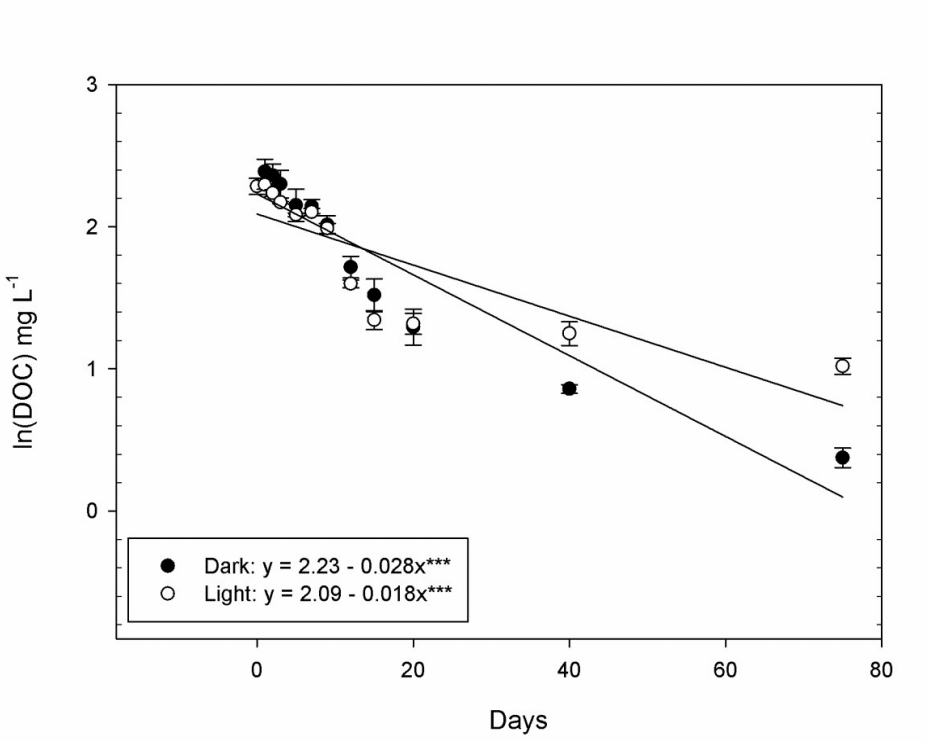

Figure 4.2. Decay rate of DOC in DU under dark and light conditions.

n= 4. *** p<0.0001. ● represents urine under dark; ○represents urine exposed to light. Error bars show standard deviation of the mean.

4.3.3 Total nitrogen (N_{tot}) and Ammonium-N (NH_4-N)

Changes of N_{tot} and NH_4-N concentrations during the experimental phase are shown in Figure 4.3. Initial N_{tot} concentration was 1.86 g L^{-1}, while NH_4-N was 1.69 g L^{-1}. This suggests that already at day 0 almost 90% of the total nitrogen of DU was found as ammonium. Depletion of N_{tot} and NH_4-N occurred in both treatments. After 75 d, 71% and 82% of the N_{tot} initial concentration were lost in dark and light, respectively. NH_4-N decreased by 65% and 81% in dark and light, respectively.

Estrogenic activity showed a significant positive correlation to both N_{tot} (r=0.399; p<0.05; n=32) and NH_4-N (r=0.357; p<0.05; n=32) in DU under dark. The same trend was found in DU under light for both N_{tot} (r=0.523; p<0.05; n=32) and NH_4-N (r=0.418; p<0.05; n=32).

4.3.4 pH and electrical conductivity (EC)

The measured initial value of pH in DU was 9.2, which is characteristic of DU that has undergone urea hydrolysis. The pH remained relatively constant during the experimental period, reaching values of 9.7 and 9.5 in DU under dark and light conditions, respectively. Regarding EC, the initial value measured in DU was at 14.7 mS cm^{-1} and decreased over time to 8.8 mS cm^{-1} in the dark and 8.1 mS cm^{-1} in the light.

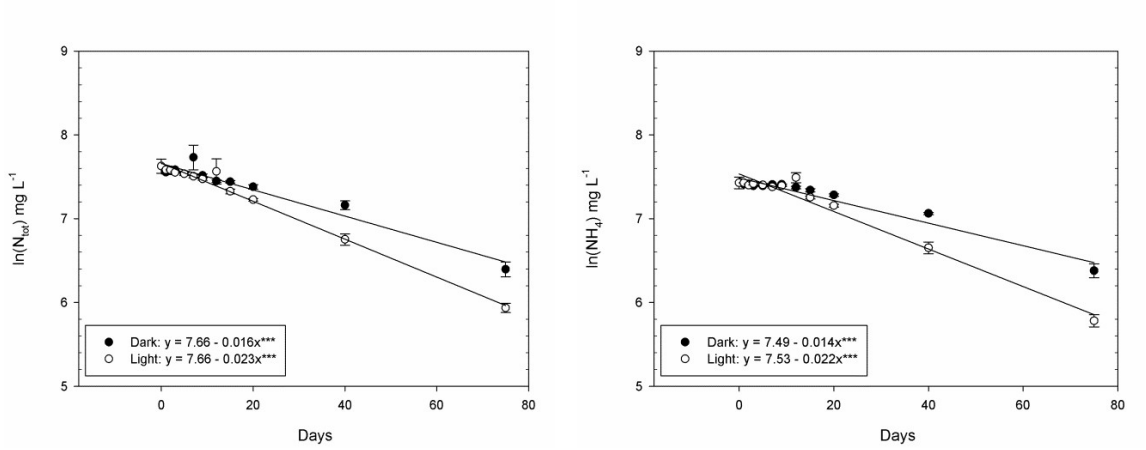

Figure 4.3. Decay of Ntot (left) and N-NH_4 (right) over time.

n= 4. *** p <0.0001. ● represents urine under dark; ○represents urine exposed to light. Error bars show standard deviation of the mean.

4.4 Discussion

In this study, a reduction of estrogenic activity was observed after 20 d in DU exposed to light as well as in DU under dark conditions. Though the decay of estrogenic activity over time appeared to be similar for both treatments, a modest increase of the reactions in DU under light occurred between day 15 and 20. This explains why the degradation rate was higher under light than in dark conditions.

According to data from DU under light corresponding to days 40 and 75, the estrogenic activity was at a zero level. However, concentration values of repetition samples at day 40 varied considerably resulting in a high standard deviation. Likewise, values from day 75 were found much lower than the detection limit. Therefore, these data were not considered in the statistical

analysis in order to avoid erroneous conclusions. In the case of DU in dark conditions, data suggested a substantial increase in the estrogenic activity after day 40. Though such increase can occur, for the purpose of comparison of estrogenic activity between the two treatments it was decided to use data until day 20. The possible causes of an increase in estrogenic activity will be discussed below.

Other studies have investigated the degradation of natural and synthetic estrogens under simulated and natural sunlight (see Table 4.1). However, to our knowledge there is no study addressing the degradation of estrogens in diluted urine. Results shown in Table 4.1 suggest a more efficient photodegradation of estrogens in a water matrix as compared to the results in the present study. Yet the present results may suggest that a longer storage period of DU under light would result in a total decay of estrogenic activity.

Table 4.1. Results obtained in this study and previous studies evaluating photodegradation of natural and synthetic estrogens.

Estrogen	Initial conc.	Matrix	Light spectrum	$t_{1/2}$	Reference
Natural excreted estrogens	2.41 µg L-1	Diluted urine	280-700 nm	10.05 d	This study
E1	100 µM	Water	290-700 nm	7.9 h	Caupos et al. 2011
		Water + fulvic acids		5.3-7 h	
E1	10 µg L-1	Water	315-400 nm	3.25 h	Coleman et al. 2004
E2				1.13 h	
EE2				0.38 h	
E1	5 µg L-1	Water	290-700 nm	0.86 h	Choudhury et al. 2010
E2	5 µg L-1	Water	290-700 nm	10.63 h	Chowdhury et al. 2011
E1	100 mg L-1	Water	Direct solar	55 d	Fonseca et al. 2011
E2			radiation	60 d	
E3				40d	
EE2				75 d	
E2	270 µg L-1	Water	290-720 nm	13.6 h	Leech et al. 2009

The fraction of dissolved organic matter (DOC) has been suggested to enhance the photodegradation of different pollutants in a water matrix (e.g. Canonica et al. 1995) as well as of steroidal estrogens, acting as a photosensitizer (Chowdhury et al. 2011; Leech et al. 2009). However, a high concentration of DOC can also hinder the degradation. Leech et al. (2009) found that a content of DOC higher than 15 mg L^{-1} saturates the solution and reduces the photodegradation of βE2, by blocking the light transmittance, and thus the chance of photons to interact with the organic compounds. In this study DOC was found in high concentrations (close to 1 g L^{-1}) at the beginning of the experiment. This suggests that during the first days of the experimental period, direct photolysis of estrogenic compounds was probably limited due to the elevated DOC content in DU. Over time, the DOC degraded exponentially in both treatments and a correlation was found between the degradation of both estrogenic potency and DOC. The reduction of DOC might be a result of light incidence, microorganism activity to obtain carbon as

an energy source and loss of CO_2. In several studies on natural waters (e.g. Wetzel et al. 1995), natural light has been proven to break down dissolved organic matter into smaller molecules. In this study, it was observed that DU became clearer after day 15, probably by precipitation of organic matter, phosphates from urine and metal ions from urine and flushing water (Höglund et al. 2000). It is hypothesized that the transmittance of light was facilitated by the reduction of turbidity in the DU and thus the interaction of photons and estrogen molecules. Therefore, estrogens in DU exposed to light could undergo direct photolysis when DOC was depleted. Since reliable values of estrogenic activity could not be obtained after day 20, future experiments may focus on longer-term degradation processes.

According to the current results, the decay of estrogenic activity was positively correlated to ammonium decay in both treatments and this correlation was found higher in DU exposed to light. However, this result might be an external influence rather than an interaction of both parameters. Decay of urea, or urea hydrolysis ([NH_2 (CO) NH_2 + $2H_2O$ → NH_3 +NH_4^+ + HCO_3^-]), results into ammonia and bicarbonate accompanied by an increase of pH. Due to the high pH, up to one third of the total ammonia consists of volatile ammonia (Udert 2002). Thus it is believed ammonium/ammonia levels were lost over time (~65%), probably by dissipation through the lids. Wohlsager et al. (2010) explained that DU stored open resulted in a 90% N loss while in closed storage, 93% of N was retained.

Previous studies suggested that pH values of 7 - 8 can enhance the direct photolysis of E1, βE2 (Chowdhury et al. 2011; Zhang et al. 2007) and the synthetic estrogen EE2 (Liu et al. 2003a; 2003b). In this study, the pH remained constant during the experimental period in both treatments (9.2 - 9.5) and there was not any correlation to the decay of estrogenic activity in any of the treatments (data not shown). As explained above, the pH in DU increases (>9) as a result of urea hydrolysis into ammonia, hydrogen carbonate and formation of OH^- ions.

Over time, the EC of urine was reduced under both, light and dark conditions. This can be a result of reactions leading to the formation of particles and sedimentation. Höglund et al. (2000) found higher concentrations of phosphate, calcium and magnesium in the bottom sediments of urine storage tanks, in the form of metal-phosphates and metal-hydroxides.

The present results showed that the decay of estrogenic activity in DU under dark was only moderately lower than in DU under light. It was assumed that the decay of estrogenic compounds in this treatment was caused by microorganisms. Though urine is sterile in the bladder, after excretion many different microorganisms may grow in the DU (Madigan et al. 2003), and cross-contamination with fecal bacteria can occur (Schönning et al. 2002; Wohlsager et al. 2010), especially in our toilet system with spill over from the feces flushing (see above).

Biotic degradation has been suggested as one of the most effective ways for removal of estrogenic compounds in fresh water systems (Jürgens et al. 2002; Matsuoka et al. 2005) and wastewater treatment plants (Fujii et al. 2002; Yoshimoto et al. 2004). Efficiency and rate of degradation of estrogenic compounds may depend on the diversity and density of microorganisms. In the present experiment with DU, the load of microorganisms most probably has been lower as in a wastewater treatment plant, and thus the reactions may have been slowed down.

Furthermore, the densities of microbial populations could have also been affected by DU conditions (such as increased pH and ammonium concentrations) as reported by Schönning et al. (2002) and Wohlsager et al. (2010). Nevertheless, Höglund (2001) reported that *E.coli*, among other enteric and opportunistic pathogens, had a longer survival time in DU diluted at a ratio of 1:9 than in undiluted urine. In our case, DU had a dilution rate of ~1:8.

As mentioned before, data corresponding to day 40 and 75 in DU under dark showed an increase of estrogenic activity (Figure 4.1). It is hypothesized that this is due to hydrolysis of conjugated estrogens. In both, male and female urine, most of the excreted estrogens are conjugated and biologically less active, mostly by esterification by glucuronide and/or sulfate groups. The esterification is a biological mechanism to detoxify free estrogens and render them more soluble and readily excreted (Khanal et al. 2006). As conjugated estrogens reach the sewer system, they can be split into free estrogens by microorganisms producing β-glucuronidases and/or arylsulfatases (Gomes et al. 2009; Khanal et al. 2006; Ternes et al. 1999). For example, β-glucuronidase activity is found in bacteria such as *E.coli* (Dray et al. 1972). Therefore, as conjugated estrogens do not exhibit estrogenicity, hydrolysis of conjugated estrogens into free estrogens might result in an increase in total estrogenic activity. In a scenario of higher rates of estrogen excretion (e.g. excretion from pregnant women, women under hormonal contraception treatment) and higher amounts of urine collection, hydrolysis of estrogens may take longer, probably leading to a longer period for reduction of estrogenic activity. However, urine collected from a bigger group of individuals may lead to a higher density and diversity of microorganisms which may also increase decay efficiency.

Another process that could explain the increase in estrogenic activity is the interconversion of E1 into βE2, as has been demonstrated by Järvenpää et al. (1980). By an *in-vitro* experiment, the authors found that the intestinal microorganisms *Alcaligenes faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus* were able to convert βE2 into E1 and *vice versa*, while *Micobacterium smegmatis* could only reduce E1 to βE2. Since βE2 has the highest estrogenic potency compared to E1 (βE2 = 1 βE2 EQs; E1 = 0.1-0.2 βE2 EQs) (Khanal et al. 2006), the accumulation of βE2 over time would result in an increase of estrogenic activity.

In this study, the YES assay was used to detect the total estrogenic potency in DU during the experimental period, independent of the type of compounds in the sample (parent or intermediate degradation products). It could not be assessed whether the aforementioned microorganisms were present in DU nor follow individual reactions; specific analytical methods have to be applied to follow the fate of individual estrogenic compounds under our experimental conditions.

4.5 Conclusions

It was shown that degradation of estrogenic compounds in DU occurs under both, dark and light conditions. Yet, DU stored in the dark may require a longer period for the elimination of estrogenic activity. In practice, with continuous load of urine into the tanks, hydrolysis of conjugated estrogens is occurring constantly. Therefore, estrogenic activity may not be reduced until urine or DU collection is stopped. Considering the $t_{1/2}$ times of estrogens, storage time of ~6 months proposed by previous studies (Höglund et al. 2002; Vinnerås et al. 2008) may be sufficient not only for the reduction of pathogens but for estrogenic activity as well. In any case, it would be important to understand the equilibrium rate of hydrolysis versus degradation of estrogens, to know how fast hydrolysis and subsequent degradation occur.

To be able to predict the remains of estrogenic compounds in DU, both, free as well as conjugated forms of estrogens need to be analyzed to get a full assessment on their fate during storage and after application. Only following the free estrogens might lead to misinterpretations as long as not both conjugated and free estrogenic activity is assessed.

This study found an efficient and inexpensive way of eliminating estrogens in DU by exposure to sunlight. However, open storage, where sunlight could hit directly the surface of the DU is definitely not recommended due to the high losses of ammonium. Thus, UV irradiation in closed tanks would be required. It should be considered as well that after application of DU onto the field, degradation of estrogens will occur due to direct sunlight exposure and higher diversity and density of microorganisms in the soil environment, as well.

Complementing the YES assay with specific analysis of free and conjugated estrogenic compounds will be essential. It might be difficult, though, to follow the fate of estrogenic compounds in more complex matrices.

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5 Plant uptake of pharmaceuticals applied by urine fertilization: The case of 17β-estradiol, carbamazepine and verapamil

5.1 Introduction

Several studies indicate that pharmaceuticals, personal care products (PPCPs), and endocrine disrupting compounds (EDCs) can be taken up by crop plants fertilized with wastewater or biosolids (Reshaw et al. 2008; Jones-Leep et al. 2010; Wu et al. 2010; Sabourin et al. 2012; Cortés et al. 2013; Dodgen et al. 2013). The uptake of PPCPs and EDCs has been verified after application of different sources of nutrients (e.g. animal manure, biosolids, wastewaster) either in soil (Boxall et al. 2006) or hydroponic systems (Herklotz et al. 2010; Shenker et al. 2010; Zhang et al. 2013). Yet little has been tested on PPCP and EDC residues excreted in urine and their transfer from soils to plants after field application (Winker et al. 2010). Before human urine can be recycled on a routine basis, the fate of PPCPs and EDCs has to be assessed to avoid undesirable contaminations in the food chain of humans and animals.

This study is investigating the potential for plant translocation of selected PPCPs and EDCs in hydroponic and soil-plant based systems. The following compounds used in this study are excreted in human urine: 17β-estradiol (βE2), carbamazepine (CBZ) and verapamil (VER). 17β-estradiol is a naturally produced reproductive hormone and is excreted by women and in lower amounts by men. Johnson et al. (2000) reported that 1.6 μg d^{-1} of βE2 can be excreted by males, 3.5 μg d^{-1} by menstruating women, 2.3 μg d^{-1} by menopausal women, and 259 μg d^{-1} by pregnant women. βE2 is very abundant in water bodies and it is considered to cause estrogenic disruption in aquatic organisms (Hamid and Eskicioglu 2012).

Carbamazepine is an antiepileptic drug used for the treatment of seizure disorders and relief of neuralgia, as well as a wide variety of mental disorders (National Center for Biotechnology Information, 2018). It is widely used and it has been found to be highly recalcitrant to elimination by the sewage treatment (Ternes 1998) and to biodegradation in the soil (Walters et al. 2010). Winker et al. (2008) who analyzed diluted urine samples from a group of source-separation toilets in the WWTP (Stahnsdorf) in Berlin and a male public urinal in Hamburg, reported a concentration of 9 and 24 μg L^{-1} of CBZ, respectively.

Verapamil is a calcium-channel antagonist that is widely prescribed and used for the treatment of supraventricular arrhythmias, coronary heart disease, antianginal therapy, myocardial ischemia and arterial hypertension (Singh et al. 1978). It is known to have immunosuppressive

(Blaheta et al. 2000) as well as cytotoxic effects (Häussermann et al. 1991) which increases the concern regarding its environmental impact, through toxicity and bioaccumulation. So far, there is no literature data on the excretion rates of VER in urine. Nevertheless, Hummel et al. (2006) monitored the concentration of VER in a sewage treatment plant in Germany, with concentrations of 3.1 μg L^{-1} in the influent and 0.51 μg L^{-1} in the effluent.

The uptake of the natural hormone βE2 has been tested in maize seedlings under hydroponic conditions (Card et al., 2012). Uptake of carbamazepine and ibuprofen has been studied in ryegrass after urine fertilization (Winker et al. 2010). Uptake of CBZ has been also tested with cucumber (Shenker et al. 2010), soybean (Wu et al. 2010) and cabbage (Herklotz et al. 2010). Uptake of VER into plants has not yet been studied.

This study consists of two different experimental settings. The first one is a hydroponic system that included assessing degradation (without plants) of βE2 and evaluation of root uptake (with plants). The hydroponic system did not use diluted human urine for nutrient supply. The second experimental setting is a soil-plant pot system used for evaluation of plant uptake of βE2, CBZ and VER applied by diluted urine fertilization.

5.2 Materials and methods

5.2.1 Chemicals

17β-estradiol (βE2) (purity ≥98%), carbamazepine (CBZ) (purity ≥99%) and verapamil (VER) (purity ≥99%) were purchased from Sigma-Aldrich, Germany. Further properties of test compounds are shown in table 5.1.

Table 5.1. Physicochemical properties and use of test compounds

Compound	Application	Chemical formula	Molar mass (g ml-1)	log Kow
17β-estradiol (βE2)	Reproductive hormone	$C_{18}H_{24}O_2$	272.4	3.1-4.0(a)
Carbamazepine (CBZ)	Anticonvulsant	$C_{15}H_{12}N_2O$	236.2	2.45(b)
Verapamil (VER)	Antihypertensive Ca- channel blocker	$C_{27}H_{38}N_2O_4$	454.6	3.79(c)

a Lai et al. 2000; b SRC PHYSPROP Database; c Hummel et al. 2006

5.2.2 Degradation and root uptake of βE2 in a hydroponic system

The degradation rate of βE2 was tested in a nutrient solution without plants for 15 d which is the time before the nutrient solution has to be renewed in the presence of plants. For this purpose, four double walled plant culture pots (Kick/Brauckman pots by STOMA, Germany) were filled with 8 L nutrient solution (Table 5.2) and βE2 was spiked as 12.5 μg L^{-1}. Oxygen was

supplied for 20 min every hour as standard in experiments with plants. Every third day (day 0, 3, 6, 9, 12 and 15), 100 ml of solution from every pot was collected and stored at -4°C until analysis.

Table 5.2. Nutrient solution used in the hydroponic system.

Compound	g L^{-1}
2M $Ca(NO_3)_2$	944.6
2M KNO_3	202.22
0,8M KH_2PO_4	109.52
0,8M $MgSO_4 * 7H_2O$	197.184
$MnCl_2*4H_2O$	3.746 (0.65 ppm)
$ZnSO_4*7H_2O$	0.704 (0.1 ppm)
$CuCl_2*2H_2O$	0.215 (0.05 ppm)
H_3BO_3	4.572 (0.5 ppm)
$Na_2MoO_4*2H_2O$	0.202 (0.05 ppm)
Fe	33 EDTA (5 ppm)

For determination of βE2 root uptake, seeds of wheat (*Triticum* L) were germinated in a sand bed, and five seedlings per pot were used. Every seedling was inserted into a foam ring which was placed into each of five holes of the pot covers (Figure 5.1). Treatments were as follows: H0 = Nutrient solution without βE2; H1 = 12.5 µg L^{-1} added at day 0 and 2.5 µg L^{-1} at each time the nutrient solution was replaced; H2= received 12.5 µg L^{-1} throughout. Every treatment had 4 replicates.

Figure 5.1. Hydroponic system.
Left: Wheat seedlings. **Right:** View of wheat root seedlings.

The experiment was run for 45 days. 100 mL nutrient solution was sampled per pot at days 15, 30 and 45 before renewing the nutrient solution. pH, EC and T°C of the nutrient solution were measured twice a week. Development and growth of the plants were monitored during the experiment by recording height (cm), fresh weight (FW g) and number of tillers per plant. At day 45, the number of spikelets per plant was registered and the wheat plants were harvested, separated into roots and aboveground biomass. Roots were thoroughly washed with distilled

water. Plant material was oven dried at 50°C for 4 d. After that, dry weight (DW g) was measured and samples were ground in a disk mill and preserved at -20°C, as well as the nutrient solution samples.

5.2.3 Uptake from a soil-plant system

In this experiment, the uptake of βE2, CBZ and VER by wheat and sunflowers grown in soil and fertilized with diluted human urine (DU) was tested under greenhouse conditions.

The topsoil of a Luvisol derived from loess was used as a test substrate (9 kg Pot). Further characteristics of the soil are presented in Table 5.3. The experiment was carried out in double walled plant culture pots (Kick/Brauckman pots, STOMA, Germany). The bottomless inner pot allowed watering through from the bottom (Kick and Große-Brauckmann 1961).

Diluted human urine (DU) was collected from a private household. A more detailed description of the collection system is given in Chapter 4, section 4.2.1. The donors, a male and female couple, had no further intake of pharmaceuticals or additional estrogens. Storage of the DU had been ~6 months after the collection time. Physico-chemical parameters of DU are shown in Table 5.3. Diluted human urine was added to the soil to achieve 1.5 g N, 0.75 g P and 2 g K in 9 kg of soil using two rates: 300 mL mixed thoroughly to the soil before sowing and 200 mL diluted in 1 L water, applied before the plants entered the blooming stage.

Before mixing with the soil, the DU was spiked with test compounds as follows: βE2 was spiked at a rate of 1μg kg^{-1} of soil (T1βE2) and 10 μg kg^{-1} of soil (T2βE2). Treatment TDU consisted of diluted urine (DU) application only. The additional estrogenic concentration added through DU (0.02 βE2-EQs μg kg^{-1}) and found in soil (0.05 βE2-EQs μg kg^{-1}) was considered in the later βE2 plant uptake calculations. CBZ and VER were spiked into the DU at a rate of 10 μg kg^{-1} of soil in T1CBZ and T1VER, respectively; and 110 μg kg^{-1} of soil in T2CBZ and T2VER, respectively. Experimental treatments were tested in sunflowers and wheat and were run with 6 replicates.

Per pot, six summer wheat (*Triticum* L) seeds and five sunflower (*Helianthus annus* L.) seeds were directly sown into the soil. After germination and full expansion of the first true leaf, plants were thinned to five and four plants per pot, respectively (Figure 5.2). The pots were irrigated with distilled water, to maintain a level of 70% of soil water holding capacity. This was achieved by watering every pot to a desired weight, after having determined the total weight of the 9 kg soil saturated to 100% with water. Watering was always done from the bottom to avoid leaching and accumulation of water soluble nutrients at the bottom of the pots.

Table 5.3. Characteristics of Meckenheim topsoil and chemical properties of diluted urine

Meckenheim topsoil		Chemical properties of diluted urine	
Clay %	16.0	N_{tot} (g L^{-1})	5.16
Silt %	77.2	NH_4^+-N (g L^{-1})	4.4
Sand %	6.9	K (g L^{-1})	4.2
Total N %	0.078	Total –P (g L^{-1})	0.29
C_{org} %	0.75	EC (mS cm^{-1})	27
pH	6.1	pH	8.68
CAL-P_2O_5 (mg kg^{-1})	35	βE2 EQs (µg L^{-1})	0.42
CAL-K_2O (mg kg^{-1})	56		
MgO (mg kg^{-1})	171		
βE2 EQs (µg kg^{-1})	0.05		

Figure 5.2. Soil-plant system.

Left: Seedlings of wheat . **Right:** seedlings of sunflower .Both planted in soil supplemented with diluted human urine.

The first harvest was after 43 d of sowing. Wheat plants were at the early booting stage and sunflower plants were at the beginning of inflorescence emergence. Only two pots of each treatment were collected. The second harvest was after 112 d for sunflower and 127 d for wheat. At this later date, the remaining 4 pots per treatment were harvested. In both harvests sunflower plants were separated into roots, stems, leaves and seeds (second harvest). In the case of wheat, the aboveground biomass in one pot was collected as a complete sample and the grains were separated from the shoot. Fresh weight was determined. Plant samples were lyophilized thereafter. Dry weight was measured for plant samples. A composite sample of soil was collected, approximately 200 g from each pot. The soil was first air dried for 2 days and thereafter crunched into smaller pieces by a wooden cylinder. The soil was subsequently oven dried at 30°C to a constant weight. Soil and plant samples were later milled using a SIEBTECHNIK® disc mill. Soil was passed through a 300 µm sieve.

5.2.4 Extraction and analysis of test compounds

17β-estradiol

17β-estradiol in the nutrient solution was extracted as described in Chapter 3 for diluted human urine. Extraction of the same compound from soil and plant material is also described in Chapter 3. However, some modifications were made for extraction of wheat grains, where 15 min of ultrasonication was followed by 15 min of shaking in a water-bath orbital shaker. Considering the difficulties encountered for extracting βE2 from plant material, which was already explained in Chapter 3, the present study added a solid phase extraction step in order to reduce the lipids, wax and chlorophyll in plant samples. For this purpose, 10 mL of the supernatant obtained after centrifugation were used for SPE column extraction. This step is described in the section for extraction of diluted human urine in chapter 3.

Analysis of βE2 in the nutrient solution, soil and plant extracts was performed by the recombinant Yeast Estrogen Screen assay (YES) described in more detailed in Chapter 3. The concentration of estrogenic activity in the samples is given as equivalents of βE2 (βE2-EQs) in μg L^{-1} or μg Kg^{-1} DW (dry weight).

Carbamazepine and verapamil

Extraction of CBZ and VER was done following the protocol established by the Zentrallabor Chemische Analytic, Technische Universität Hamburg-Harburg (TUHH) for extraction of pharmaceuticals in cereals. The detailed protocol is shown in the Annex 3. The extraction procedure is briefly described below:

Solid-liquid phase extraction: two repetitions of 5 g of soil or plant sample were placed in a 50 ml centrifugation tube with 20 mL of MeOH/citric EDTA buffer solution (10/90 v/v) and were mixed by hand until homogeneous. Tubes were placed in the ultrasonic bath (Bandelin SONOREX SUPER RK 102H), at a frequency of 35 khz and at a constant temperature of 30°C for 5 min. Afterwards, the tubes were agitated at 225 (rpm) for 60 min. Later, the samples were centrifuged for 10 min at 6000 rpm.

Solid phase extraction (SPE): SPE columns CHROMABOND® EASY polar modified polystyrene-divinylbenzene copolymer 6mL / 200 mg (Macherey-Nagel) were attached to a Tall Boy™ - 10 position Vacuum manifold (Phenomenex). The columns were activated with 5 mL 100% MeOH and then washed with 5 mL 5% MeOH. The sample extract (supernatant) obtained from the solid-liquid phase extraction was then transferred with a Pasteur pipette onto the SPE column and left to drain (30-40 drops /min). After this, the SPE columns were blown dry under nitrogen gas for 30 min. and compounds were eluted with 10 ml of MeOH 100%, followed by 10 ml of

MeOH + 0.1% acetic acid. The SPE columns were dried again under nitrogen gas. Solid phase extraction was repeated with the residues left in the centrifugation tube. This time 20 ml of MeOH/boric acid buffer (10/90) were added and mixed thoroughly by hand. Tubes were agitated for 30 min. Extract was transferred to the same SPE column used initially and combined extracts were transferred to a 50 ml round bottom flask.

Sample concentration: The extract was reduced to approximately 1 mL in a rotary evaporator (BÜCHI Rotavapor R-124 at 80-100 rpm; BÜCHI Waterbath B-480 at 45-50°C; BÜCHI Vac® V-500). The remaining solution was then transferred into a 2 ml vial and filled with MeOH up to the mark. The sample was left to settle overnight in the fridge and then carefully transferred into a microcentrifuge tube to avoid mixing the sediments. Samples were centrifuged in a Biofuge for 10 min at 13000 rpm. The supernatant was collected into brown glass autosampler vials for analysis.

Carbamazepine and verapamil were analyzed according to Dalkmann et al. (2012) by using liquid chromatography tandem mass spectrometry (LC-MS/MS). The method is described in detail in the supplementary information (Text S2) in Dalkmann et al. (2012). The limit of detection (LOD) for CBZ and VER are 6 ng kg^{-1} DW and 14 ng kg^{-1} DW, respectively. The limit of quantification (LOQ) for CBZ and VER are 22 ng kg^{-1} DW and 42 ng kg^{-1} DW, respectively. Both limits are for soil and plant parts.

5.2.5 Statistical analysis and other calculations

Statistical analysis was performed by using IBM® SPSS® Statistics software (Version 21). The compounds concentration in nutrient solution and the soil and plant material is presented as mean ± standard deviation of replicates. One and two way ANOVA (significance level = 0.05) was used to determine significant differences between treatments of both experiments.

The bioconcentration factor (BCF) describes the ratio of relative uptake of each compound in plant tissues to the concentration of each compound spiked in soil (See Equation 1).

Equation 1 $$BCF = \frac{\text{Compound concentration in dry plant tissue } (\mu g\ kg^{-1} \text{ or } \mu g\ L^{-1})}{\text{Compound concentration spiked in matrix } (\mu g\ kg^{-1} \text{ or } \mu g\ L^{-1})}$$

The concentration and bioconcentration factor were calculated based on dry weight (DW).

5.3 Results

5.3.1 Degradation and root uptake of βE2 in a hydroponic system

The degradation of βE2 in the hydroponic system without plants resulted in 98% over 15 d. The linear plot of $ln(C_{\beta E2\ EQs}/C_{0\ \beta E2\ EQs})$ versus time (Figure 5.3) shows the decay of βE2 as a first-order reaction. The first order degradation rate constant was calculated by the following equations 2 and 3:

Equation 2 $\ln (C_{E2EQs} / C_{0\ E2EQs}) = -kt$

Equation 3 $t_{1/2} = \ln 2 / k$

where $C_{0\ \beta E2\ EQs}$ and $C_{\beta E2\ EQs}$ are the concentration of estrogens at time zero and after, k is the reaction rate (d^{-1}) and $t_{1/2}$ or half-life refers to the time in days needed to degrade 50% of the initial concentration. The degradation reaction rate k was calculated as 0.27 d^{-1} and the half-life ($t_{1/2}$) of βE2 was 2.6 d in the hydroponic system without plants.

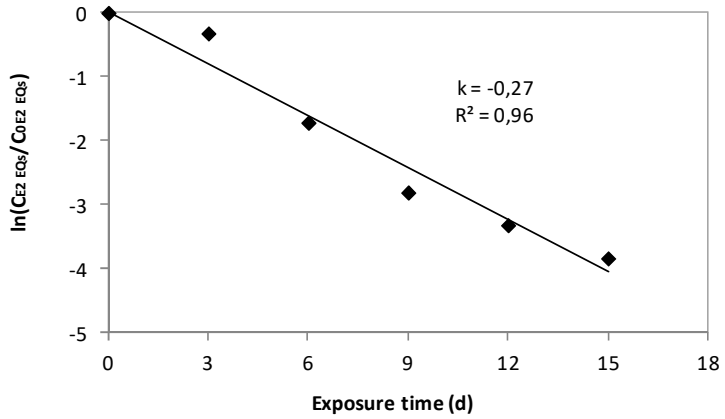

Figure 5.3. Degradation of βE2 in hydroponic system.
k=constant reaction rate. Initial concentration: 12.5 µg L^{-1} βE2 EQs.

The detection limit was calculated based on the volume of the sample used for extraction. As it was assumed that the concentration of βE2 was decreasing over time, the extracted volume was increased. Thus, the sample volumes were increased from 5 to 40 ml. In Table 5.4, the different DL values are listed.

Table 5.4. Detection limit (DL) of βE2 for the corresponding sample volume

Day	Extracted sample volume	LOD ng L^{-1} βE2-EQs
0	5	28
3	10	14
6	20	7
9	40	3.5
12	16	8.7
15	32	4.4

Figure 5.4 shows the concentration of βE2 in the roots and in the nutrient solution for each 15 d interval of solution renewal. In general, βE2 was detected in very low amounts in both the nutrient solution and the wheat roots. The concentrations remained below 0.3 µg L^{-1}. Root tissues in both treatments, H1 and H2, showed concentrations lower than 0.2 µg kg^{-1} DW βE2 EQs after 45 d. However, a slight increment of estrogenic concentration of roots was detected in treatment H2 after 45 d. The bioconcentration factor (BCF) estimated the ratio of βE2 in wheat roots as 0.02 and 0.01 for roots in H1 and H2, respectively.

Residues of βE2 found in the nutrient solution were higher at the end of the experiment (Day 45) for treatment H2. However, a high standard deviation indicated a high variability between replicates and thus no statistical significance was found. Estrogenic activity in both the roots and the nutrient solution was found in treatment H0 without any application of βE2. This concentration was very similar to treatment H1.

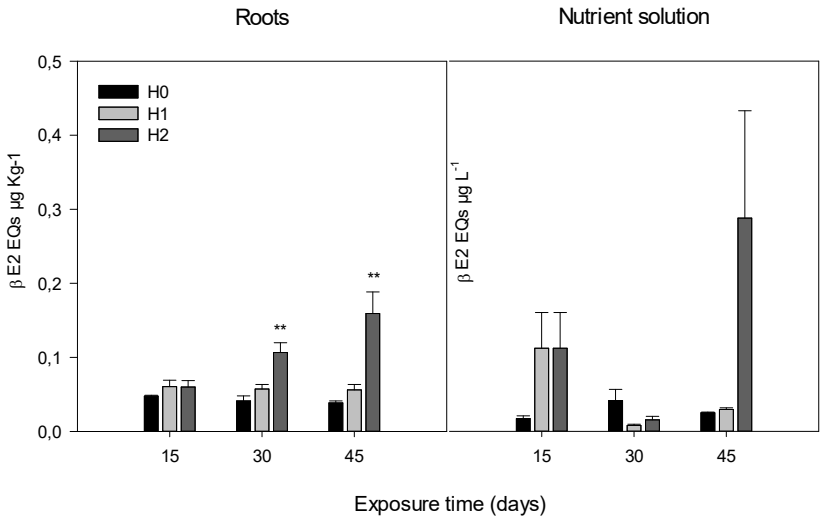

Figure 5.4. Concentration of βE2 in roots and nutrient solution.

** indicate significant differences among treatments ($p<0.05$; n=4). Error bars show standard deviation of the mean.

5.3.2 Uptake of compounds from a soil-plant system

17β-estradiol

The extraction of βE2 from soil material and wheat grains was highly efficient (Table 5.5). Extractability from sunflower stems and seeds and wheat above-ground biomass was very low. Yeast density was negatively affected in wells with extracts of roots from both sunflower and wheat as well as from leaves of sunflower (Figure 5.5). However, only extracts of wheat roots obtained at first harvest showed yeast inhibition. In those cases, absorbance values were as low as 0.1-0.3 at a wave length of 630 nm, whereas the values should range between 0.9-1. Causes of yeast inhibition will be discussed later. The limit of detection (LOD) of βE2 was calculated for soil and roots of both crops sunflower and wheat, as well as for wheat grains as shown in Table 5.5.

Table 5.5. Recovery (%) of βE2 and limit of detection (LOD).

	Recovery (%)±STDEV	**LOD ng kg^{-1} DW**
Soil	107.6±19.5	4
Sunflower		
Roots	<0.000*	4
Leaves	<0.000*	ND
Stems	1.94±8.8	ND
Seeds	-0.40±2.0	ND
Wheat		
Roots	<0.000*	4.17
Aboveground biomass	0.73±0.16	N.D
Grains	96.2±14.3	2.5

(ND = not determined). *yeast inhibition; STDEV: standard deviation of the mean.

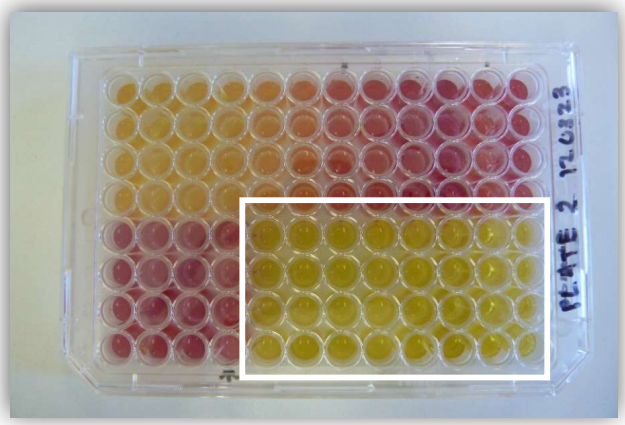

Figure 5.5. Microtitre plate with YES assay after incubation.

Upper four rows correspond to the standard samples (2 – 2700 ng L^{-1}). Lower four rows correspond to samples. Squared region shows no color change to red as a result of yeast growth inhibition in sunflower.

Estrogenic concentration found in soil and plant tissues of sunflower and wheat from first and second harvest is shown in Figure 5.6. Only data from soil and roots of both, wheat and sunflower treatments in harvest 1 and 2, and wheat grains are shown. Analysis of variance was performed only for data collected at second harvest (n=4). The concentrations of βE2 in soil and plant parts (where measurements were possible) ranged between 0.01 and 0.16 μg kg^{-1} DW βE2-EQs. The residual βE2 in soil of T2βE2 with sunflower culture after harvest 2, was significantly higher ($p<0.05$) than T1βE2 and TDU. In contrast, residues in soil with wheat at harvest 2 were very low for all treatments. In the roots of the sunflowers, βE2 was found in higher amounts in the samples from the first harvest as compared to the second harvest. However, the concentrations in roots of sunflower from TDU were higher for the second harvest compared to first harvest. Concentrations detected in wheat grains were low throughout and not significantly different between treatments.

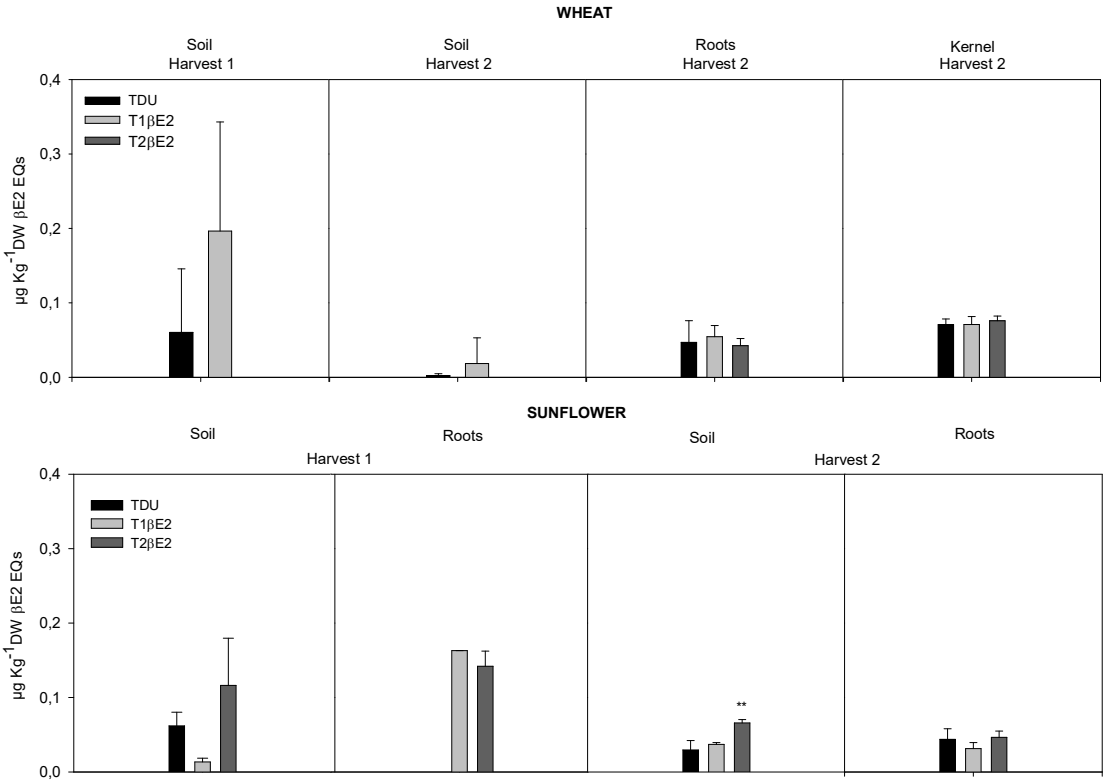

Figure 5.6. Mean concentration of βE2 in soil and plant parts of wheat and sunflower.
TDU=0.02 μg kg^{-1}; T1βE2=1 μg kg^{-1}; T2βE2=11 μg kg^{-1}. Error bars show standard deviation of the mean (first harvest n=2; second harvest n=4). ** indicate significant differences among treatments ($p<0.05$; n=4)

The BCF of βE2 in the roots of sunflowers and wheat, as well as wheat grains is shown in Table 5.6. In general, the BCF indicates that the concentration ratio of βE2 was low in the referred

plant parts of sunflowers and wheat for both treatments, T1βE2 and T2βE2. However, the values indicate a high BCF for TDU at second harvest in roots of sunflowers and wheat as well as in wheat grains which showed the highest BCF value.

Table 5.6. Bioconcentration factors (μg kg^{-1} DW) of βE2 in sunflower and wheat.

	First Harvest (43 d)		Second harvest ([a]112 d; [b]127 d)		
	T1βE2	**T2βE2**	**TDU**	**T1βE2**	**T2βE2**
[a]Sunflower					
Roots	0.15	0.01	1.88	0.03	0.00
[b]Wheat					
Roots			2.01	0.05	0.00
Grains			3.03	0.06	0.01

In general, ratios of βE2 recovered from soil and plant material (where measurements were possible) were very low. In wheat culture (soil, roots, and grains), only 1% of the total βE2 applied to each treatment was detected. In the sunflower culture, the percentage was similarly low, except for the ratio found in soil of TDU, which accounted for almost 50% of the initial amount of βE2 EQs detected in urine and soil together. During the experimental period, there was no evidence of negative effects of the βE2 application on growth and/or development of wheat and sunflower plants in the different treatments.

Carbamazepine and verapamil

Due to financial constraints, the analysis of CBZ and VER were performed only for soil and aboveground biomass of both wheat and sunflowers corresponding to T2CBZ and T2VER of the second harvest. The roots were not analyzed.

Both, CBZ and VER residues were found in soils as well as in wheat and sunflower tissues (Table 5.7). However, analysis showed that CBZ was found at a considerably higher rate in both culture systems, as compared to VER. A high proportion of CBZ was accumulated in the aboveground biomass of wheat and in the leaves of the sunflowers. Residues of CBZ in wheat culture were significantly higher ($p<0.05$) in soil and aboveground biomass than in the grains. Whereas in sunflowers, no significant differences were found. A high variation was found in the standard deviation between sunflower leaves. Residues of VER were high in soils of both wheat and sunflower cultures. However, in soils of wheat culture, the concentration was significantly higher ($p<0.05$) than in the plant parts. VER was not detected in the stems of the sunflowers. No significant differences were found for total concentration of both, CBZ and VER in wheat and sunflowers as representatives of monocotyledones and dicotyledones.

Table 5.7. Mean concentration of CBZ and VER (µg kg^{-1}) ±STDEV in wheat and sunflower.

	TDU	T2CBZ	TDU	T2VER
Wheat				
Soil	0.04±0.06	45.05±3.25	1.49±1.58	2.76±0.28
Shoots	0.09±0.10	361.06±34.03	0.23±0.46	0.83±0.09
Grains	0.12±0.14	0.99±0.18	0.03±0.04	0.03±0.03
Sunflower				
Soil	0.00±0.00	39.56±13.70	0.00±0.00	1.77±0.38
Stems	1.07±0.29	21.55±30.28	0.21±0.01	0.00±0.00
Leaves	1.33±0.24	499.18±546.20	0.20±0.04	1.52±1.55
Seeds	0.07±0.14	0.49±0.48	0.26±0.31	0.27±0.33

TDU (Diluted urine); T2CBZ (110 µg kg^{-1} of soil); T2VER (110 µg kg^{-1} of soil); n=4; STDEV: standard deviation of the mean

The soils of both the wheat and the sunflowers retained the largest proportion (40.5% and 35.6%, respectively) of the original amount of applied CBZ. In contrast, only a small proportion of VER (2.5% and 1.6%, respectively) was found in soils of the wheat and the sunflowers, respectively. Proportions of CBZ and VER in plant parts of sunflowers and wheat were close to Zero. Consequently, results suggest that approximately 60% of CBZ and 98% of VER were either accumulated on the roots or not detected in any of the cultures.

Neither visual observation nor fresh or dry weights revealed any inhibition of plant growth for plants treated with CBZ and VER.

The BCF of CBZ and VER in sunflower and wheat plant parts is shown in Table 5.7. Calculations indicated that CBZ had the highest ratio in leaves and aboveground biomass, while BCF for VER was close to zero in all plant parts of both species.

Table 5.8. Bioconcentration factors (µg kg^{-1} DW) of CBZ and VER in wheat and sunflower.

	CBZ	VER
Sunflower		
Stem	0.19	0.00
Leaves	4.50	0.01
Seeds	0.00	0.00
Wheat		
Aboveground biomass	2.44	0.01
Grains	0.01	0.00

5.4 Discussion

Methodological constraints

This study used the YES assay as a tool for the analysis of estrogenicity in plant material grown both, in hydroponic and soil system. YES has demonstrated its efficiency over other analytical tools because it is simple to handle, inexpensive and possesses high sensitivity. It has been

extensively used for analysis of waters and wastewaters with no constraints. However, monitoring the estrogenicity of plant material with this tool has been very scarce. Chapter 3 explains the procedure of the YES assay and the extraction methodologies for different materials, as well as the difficulties encountered during extraction of plant material.

In the present, study sunflowers and wheat were used as test plants. Based on the results that were experienced in Chapter 3 with the extraction of spinach, some modifications were made to the procedure. The time of ultrasonication was shortened in order to reduce the yield of lipid and wax from the plants and the solid phase extraction clean-up step was implemented to remove the excess of those substances before analyzing. Unfortunately, they could not be completely removed causing some fat and wax to remain in the flask after evaporation. The final eluted sample appeared turbid due to the tiny lipidic clumps despite double centrifugation. From the disparity of the absorbance values, it is thought that the turbidity in the samples might have interfered and therefore the results were not reliable.

Yeast density was negatively affected in wells with extracts of roots from both sunflowers and wheat (samples from first harvest) and leaves of sunflowers. There is sufficient evidence supporting that sunflowers, as well as wheat, produce biochemicals that influence the growth, survival, and reproduction of other organisms (Chavez das Neves and Monteiro Gaspar 1990; Macías et al. 1999; Wu et al. 2001; Bertin et al. 2003; Weisz et al. 2009). Among these biochemicals, lignans have been identified in numerous studies by their biological properties as, for example, antioxidant, antitumor, antiviral, antibacterial, and antifungal (MacRae and Towers 1984). Slevin (1991) has observed that some lignans can be cytotoxic and cause DNA strand brakes, such as the ones extracted for antitumoral purposes. Aehle et al. (2011) investigated the estrogen and antiestrogen activity of natural and synthetic lignans by using the YES assay. They reported that the lignan pinoresinol (1.4 mM) diminished yeast growth after five days of incubation to 71%, while daidzein and genistein were well tolerated. Pinoresinol has been reported to be present in both sunflowers (Macias et al. 2004) and wheat (Smeds et al. 2009). However, it is outside the scope of the present study to determine the concentration rate of these biochemicals (e.g. lignans) in wheat and sunflowers. Also, an additional analysis would be required to explain why younger wheat roots inhibited yeast growth more than older roots from the second harvest.

In the specific case of sunflower seeds, though the extract was mainly oil, there was not any difficulty with the elution after evaporation. Even though, during the incubation there was no color development in those samples, and the recovery % was Zero, no evidence of yeast inhibition was found. Therefore it is suggested that the oil fraction could have intervened in the

reaction process by wrapping the yeast cells, thus avoiding the contact between the estrogens in solution and the estrogen receptor (hER) in the yeast cells.

Degradation and root uptake of βE2 in a hydroponic system

Hydroponic experiment without plants showed that βE2 is readily degradable in nutrient solutions. The aerobic conditions maintained throughout the experimental period allowed bacterial colonization and proliferation which are the major cause for degradation of βE2 (Khanal et al. 2006). It is thought that a frequent oxygen supply to the system must have accelerated the degradation process.

The hydroponic system with wheat plants showed a rather low root uptake, even in treatment H2 where 12.5 μg L^{-1} was spiked every 15d for a period of 45 d. In this treatment, the average residual concentration in the nutrient solution was higher than the concentration found in the roots at the end of the experiment with levels lower than 0.3 μg L^{-1} βE2-EQs. The β17-estradiol was found in such low concentrations in root tissues that it probably did not reach the upper parts of the plant in high amounts. Furthermore, it is highly probable that the degradation rate of βE2 in the nutrient solution was higher than the root uptake rate. However, due to the methodological difficulties discussed above, there is a lack of data showing βE2 translocation into aboveground biomass.

Card et al. (2012) tested the uptake of βE2 and E1 (estrone) in maize seedlings and found that both compounds were not detected in the nutrient solution after 12d and 18d of exposure, respectively. However, compounds were detected in higher amounts in root tissues than in shoots of maize seedlings with a βE2 concentration higher than E1. The authors attributed the disparity of translocation of βE2 between root and shoot to limited translocation or to transformation and irreversible binding in root tissues.

Translocation of compounds into the aboveground biomass can be limited if those are not able to reach the vascular tissue (xylem / phloem). The challenge, after being taken up by the roots, is to move through the epidermis, the cortex, and the hydrophobic barrier of the Casparian strip located in the endodermis. Compounds could use any of these routes: moving between cells through cell walls and membranes, between cells through interconnecting plasmodesmata (symplastic), and along cell walls through the intercellular space (apoplastic). Solutes moving by an apoplastic route are not able to cross this barrier and therefore accumulate in the roots (Miller et al. 2016).

Dietz and Schnoor (2001) suggest that compounds to be transported, may have an optimum level of hydrophobicity that allows the chemical to bind to the lipid bilayer of the cell membrane

to be easily transported. Briggs et al. (1982) demonstrated that highly hydrophobic chemicals (log K_{ow} >3.5) are bound so strongly to the surface of roots and soils that they cannot be translocated easily within the plant, and chemicals that are quite water soluble (log K_{ow} <1.0) are not sufficiently absorbed to roots nor actively transported through plant membranes. According to this, and considering log K_{ow} values of βE2 (3.1 – 4.0) reported by Lai et al. (2000), the compound has a moderate to high hydrophobicity and is probably not easily transported from the roots to the upper parts of the plants.

Dietz and Schnoor (2001) also suggest that compounds can be transformed rapidly at the root surface by extracellular enzymes or by membrane-bound enzymes; however more research on that topic is required.

Uptake of 17β-estradiol from a soil-plant system

Analysis of estrogenic activity in wheat grains revealed values of β17-estradiol in the very low range (~0.07 μg kg^{-1} βE2-EQs). Interestingly, the estrogenic activity in wheat grains was not significantly different (p>0.05) between all three treatments (TDU, T1βE2, T2βE2), irrespective of the fact that the concentration applied in TDU with addition of diluted urine was 500 times lower than in T2βE2. This result suggests that estrogenicity is inherent in wheat grains and may be derived from phytoestrogens[1]. The major groups of phytoestrogens are isoflavonoids and lignans. Lignans are in higher concentration than isoflavonoids in cell walls and fibers of wheat grains and sunflower seeds (Thompson et al. 2006). Smeds et al. (2009) identified the presence of medioresinol (MED), lariciresinol (LAR), pinoresinol (PIN), secoisolariciresinol (SEC) and 7-hydroxymatairesinol (HMR) in whole-grain wheat flour.

Although, it was not possible to analyze the complete culture system (soil and plant parts) to know the relocation of the test compound βE2, it was assumed that a very low uptake occurred. The present results suggest that most of the amount applied at the beginning of the experiment could have been degraded during the experimental period. The proof is that the ratios of βE2 at the end of the experiment were as low as 1% of the total amount applied at the beginning of the experiment. Interestingly, the ratios of estrogenicity found in soils of the treatment TDU with sunflower, indicated that a ratio of almost 50% was detected at the end of the experimental period. The ratio can be considered high, however the initial concentration through diluted

[1] A phytoestrogen is defined as any plant compound structurally and/or functionally similar to ovarian and placental estrogens and their active metabolites. This definition includes compounds with agonistic, partial agonistic, and antagonistic interactions with estrogen receptors (ERs) and other targets of estrogenic steroids involved in estrogen transport, synthesis, and metabolism (Whitten and Patisaul 2001).

urine and soil accounted only for 0.07 μg kg^{-1} of soil. Such low amounts of estrogenic activity would be expected to degrade and be almost or negligible at the end of the experimental period. However, this was not the case. The data indicated the same trend in all repetitions of TDU treatment which can be attributed to the specific biochemical environment created by sunflower roots and this would negatively influence the microorganisms responsible for the degradation of estrogenic compounds.

Previous studies demonstrated that steroidal estrogens are very susceptible to degradation in soils especially by biological processes (Lucas and Jones 2006; Fan et al. 2007; Goeppert et al. 2014). Furthermore, Lucas and Jones (2006) suggested that rate of mineralization and degradation of the hormones E1 and βE2 depended on soil type and matrix in which hormones are added. In a sandy soil with very low organic content (0.2%) the highest rate of E1 and βE2 degradation was seen after addition to the soil of sheep urine while the lowest rate was observed using distilled water.

Uptake of carbamazepine and verapamil from a soil-plant system

In this study, it was confirmed the persistence of CBZ in the soil as well as its accumulation in wheat grains and sunflower seeds. In previous studies, this compound has been shown to be very persistent not only to wastewater treatments but also after application of treated sewage (Ternes et al. 2007), treated wastewater (Durán-Alvarez et al. 2009), or dewatered municipal biosolids (Gottschal et al. 2012). In the present case, three months of post-application (112 and 127 days for sunflowers and wheat, respectively) of diluted urine spiked with CBZ, caused the highest percentage of the compound to be extracted from the soils of both culture systems: sunflowers (35 %) and wheat (45 %).

In a similar experiment, Winker et al. (2010) tested the uptake of CBZ by ryegrass, using diluted urine (yellow water) as fertilizer and the same type of soil as in the present study. The authors applied CBZ at two different concentrations (3.2 μg kg^{-1} and 32 μg kg^{-1}) and recovered an average of 53% and 69% from the soils, respectively. Although the current work has applied a higher concentration of CBZ (110 μg kg^{-1} of soil DW), the accumulation in the soil was lower than reported by Winker et al. (2010). This study had a longer experimental period, though considering the persistence of CBZ, 10 to 20 d more probably did not make a significant difference in degrading the compound. Previous studies have shown the very low degradation potential of CBZ in soils (Monteiro and Boxall 2009; Grossberger et al. 2014; Walters et al. 2010).

While a relatively high proportion of CBZ was recovered in soils of both culture systems, a very low percentage was found in the upper plant tissues of both wheat and sunflower. Interestingly,

this low proportion was mostly accumulated in sunflower leaves (1.2%) and shoots of wheat (0.5%). This finding agrees with that of Winker et al. (2010) who reported that ~33% of CBZ accumulated in aerial parts of ryegrass 3 months after application, and only an average of 0.26% was recovered in the roots. Similarly, Schenker et al. (2011) have shown that the accumulation in mature older leaves of cucumbers was significantly higher than in roots and stems. Wu et al. (2010) found that leaves of soybeans showed the highest concentration of CBZ after a 60 d growing period. Mc Farlane (1995) suggested that a higher accumulation of some micropollutants in stems and leaves is related to their lipophilic nature and the large apolar surfaces of leaves (cuticules) and stems, forming a sink for apolar substances. CBZ is considered to be a moderately hydrophobic compound with an octanol/water partitioning coefficient (log K_{ow}) of 2.45. Brigs et al. (1982) suggested that there is a higher probability of movement into the plant root and translocation up the stem to the plant leaves at log K_{ow} values in the range of ~1.0-2.5.

Carbamazepine translocation into wheat caryopses and sunflower seeds has been shown in this study, though in the very low range (~1 μg kg^{-1}). Schenker et al. (2011) reported detection of CBZ in cucumber fruit grown in 3 different substrates (sandy soil, clay soil and peat mixture). Accumulation of compounds in leaves and stems does not always imply that the compound will also accumulate in fruits or seeds. This is the case reported by Gottschall et al. (2012) who did not find any accumulation of CBZ in wheat grains after application of municipal dewatered biosolids contaminated with CBZ (183 μg kg^{-1} DW). Likewise, Wu et al. (2010) did not detect CBZ in soy beans after an experimental period of 110 days. Yet accumulation in leaves may pose a risk when growing plants with edible leaves (e.g. lettuce, spinach, collard greens). This demonstrates that there are certain constraints (e.g. physical, environmental, physiological) that may limit the uptake of the compound to the generative tissues of crops.

For the first time, this study demonstrates that VER can accumulate in soils and plants, though in much lower proportions compared to CBZ. For VER, the highest recovery was found in the soil of both, wheat and sunflower systems. The recovery rates from the soils were only 2.5% and 1.6%, respectively. The compound was translocated into shoots and grains of wheat, and at a higher proportion into the leaves and seeds of sunflowers. It has to be stated that the concentrations of the compound are almost negligible. The low translocation of VER can be explained by its lipophilicity.

According to the results, VER was found in very low amounts in both culture systems which is most likely caused by microbial degradation. Trautwein et al. (2008) studied the aerobic biodegradability of VER and stated that it can only be biodegraded in an aerobic environment with a high microbial diversity and optimal nutrient supply. However, the same authors

reported that the degradation product (D617) seems to be very stable and is toxic to microorganisms or at least persistent. Thus, the compound has been classified as a dead-end metabolite. Kroemer et al. (1997) identified D617 as one of the two primary breakdown products in the first pass metabolism of VER in the human organism, which is excreted in urine and feces. Eichelbaum et al. (1979) reported that 3-4% of a dose of VER is excreted unchanged in the urine and 22% in the form of D617. In the present case, identification of degradation products was beyond the scope of the study. However it opens a new direction to study the fate of verapamil and its degradation products.

5.5 Conclusions

This research has highlighted the advantages of using the YES assay for screening estrogenicity in soil, wheat and sunflower roots, wheat grains, human urine and nutrient solution. Further efforts are required to improve the extraction methodology for using the YES assay in vegetative tissues such as leaves. However, some plant species may be difficult to analyze due to metabolites (mostly from the secondary metabolism) which may interfere with yeast development.

Based on the current experiences with the YES assay, the method has to be compared to and complemented by further analytical methods (e.g. LC/MS) to avoid misinterpretation of the data and to better understand the translocation processes into plants.

With this study, it is shown again the high degradation rates of βE2. Therefore, it is suggested that the accumulation of βE2 in agricultural field should not pose a problem since it is easily degraded via different pathways (e.g. photodegradation and biodegradation). In soils, estrogens are exposed to a great diversity and density of microorganisms which are able to degrade estrogens. At the soil surface, exposition to direct sunlight induces the photolysis of steroidal estrogens, as it does in aquatic systems, too. Consequently, the highest risk is the overload of wastewater onto agricultural land. This might induce percolation into the water bodies, where estrogens may affect aquatic organisms even at *ng* concentrations.

This study suggests that more attention should be given to persistent compounds such as CBZ that are very difficult to degrade. Its persistence in soils for longer time periods increases the chance to be taken up by plants, to percolate soils and to leach into groundwater. In the present study, where realistic concentrations were applied, the accumulation in the generative (edible) parts like wheat grains and sunflower seeds was rather low and do not reach concentrations for therapeutic use. However, more attention should be paid to monitor the consumption and

accumulation of such compounds when using human excreta on the agricultural fields as well as their transfer into the water bodies.

As compared to CBZ, VER has not been frequently studied as an emerging micropollutant and very little is known about its environmental risk. However, this study shows that it also accumulates in soils as well as in plants, and most importantly in edible parts. Although the concentrations found were very low, there is the need to investigate more the status and the degradation potential of this compound in different environmental systems (e.g. soils and natural water environments).

5.6 References

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6 General conclusions and recommendations

The present thesis was developed to identify the potential risks of loading pharmaceuticals and hormones to field crops when using human urine as fertilizer. Furthermore, it attempted to evaluate the conditions, the processes and the methods for recycling human urine in developing countries. This is of importance when recycling technologies are applied to close nutrient or material flow cycles.

The case study in Bogotá, showed the lack of a sewage system in the visited peri-urban and rural areas of the city. In some areas, wastewater runs freely on the streets as it was observed in Ciudad Bolivar. In most of these areas, wastewater is not treated before being disposed into the rivers. Therefore, there is a high risk of pollution of water resources and also a risk for human health. Many people complained about frequent intestinal infectious diseases resulting from low drinking water quality.

Furthermore, the case study showed the difficulties that the implementation of ASUs have encountered on the way to improve sanitation in rural and peri-urban areas of Bogotá. Within the project, only 6 of the 32 urine-diverting dry toilets were still in use after 5 years. Nevertheless, households that kept the ASUs showed sanitation improvement, safe management of household wastewaters, safe access to drinking water, and safe reuse of human feces and urine in agricultural fields. Importantly, the members experienced much less incidence of infectious diseases. These experiences show the potential of alternative sanitation technologies and attempts should not be stopped due to failures in the past, especially in countries like Colombia where peri-urban and rural areas are in need of improving sanitation and access to water.

In addition, this study identified antihypertensives and analgesics/anti-inflammatories as the most-consumed pharmaceuticals and its active ingredients in a population group in the locality of Suba. Due to the lack of proper wastewater treatment there is a risk that excreted pharmaceutical residues reach water bodies. In fact, other studies showed the presence of the analgesics/anti-inflammatories acetaminophen, diclofenac, ibuprofen and lidocaine; and the antihypertensives valsartan, losartan and metoprolol, among other compounds, in wastewaters and surface waters in Bogotá.

Governments and pharmaceutical industries should work towards a more sustainable approach that guarantees PPCPs and EDCs to be easily biodegradable, which would especially help regions

that lack (sophisticated) wastewater treatment. Also, proper information of handling, disposal and management of pharmaceutical compounds to doctors, pharmacists and patients should be provided in order to reduce the incidence of those compounds in the environment. Therapeutic practices should consider the effect of their treatments on the environment.

In the search of an analytical tool that was cost effective, and required low investment in facilities and equipment as compared to other analytical methods, the YES assay was tested for analysis of estrogenic compounds in soil and plant material as well as in human urine. The YES assay was found to be a reliable analytical technique for measuring estrogenic activity in soil, wheat grains and human urine. However, YES does not identify specific compounds, thus, it is recommended to complement it with another analytical method such as LC/MS.

Estrogenic potency in human urine was reduced in both under light and dark conditions. However, urine stored in the dark, may require a longer period. The degradation potential of βE2 was also evaluated under hydroponic conditions and it produced a 98% degradation in a period of 15 days, therefore, the accumulation of βE2 in an agricultural field should not be a problem since it is easily degraded via different pathways (e.g. photodegradation and biodegradation). In soils, estrogens are exposed to a great diversity and density of microorganisms which are able to degrade estrogens. At the soil surface, exposure to direct sunlight induces the photolysis of steroidal estrogens, as it also does in aquatic systems. It is seen that the highest risk is in the overload of wastewater onto agricultural land. This might induce percolation into the bodies of water where estrogens may affect aquatic organisms even at *ng* concentrations.

It is important to monitor closely persistent pharmaceutical compounds such as carbamazepine. Longer time in soils increases the chance to be taken up by plants, to penetrate deeper into the soil, and leach into groundwater. In the current study, the accumulation of carbamazepine in the generative (edible) parts like wheat grains and sunflower seeds, was rather low and did not reach concentrations that would be equivalent to therapeutic use. However, more attention should be paid to monitor the consumption and accumulation of such compounds when using human excreta on the agricultural fields as well as their transfer into the aquatic environment.

The other evaluated compound, verapamil, has not been frequently studied as an emergent micropollutant and very little is known about its environmental risk. This study showed that it also accumulates in soils as well as in plants, and most importantly, into edible parts. Although the concentrations found were very low (<3 $\mu g\ kg^{-1}$), there is the need to investigate the fate and degradation potential of this compound in different environmental systems (e.g. soils and natural water environments).

This study suggests that the direct use of human urine as fertilizer might not be as environmentally safe as first thought. The main reason relates to the risk of overloading crop fields with persistent micropollutants (e.g., carbamazepine, verapamil) that eventually could reach groundwaters. This presents a limitation for direct application of human urine. Therefore there is a need to study safest and effective methods for using the nutrients from urine to reduce the risks of pharmaceutical and hormone contamination.

However, the use of human urine as fertilizer could be practiced relatively safely at household scale gardens or small plots where the required amounts of human urine are in the lower range and the identification of persistent PPCP or EDC compounds would be easier. In this way, the impact on human beings and the environment´s health would be reduced.

Appendix 1.

Questionnaire for evaluation of medicament consumption in a population group in Bogotá, Colombia

Questionaire No_______. Locality, address____

Section I: Family members and education level

	Gender			Education level				
Member No	Female	Masculine	Age	Primary	Secondary	University	Technology	None
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐
	☐	☐		☐	☐	☐	☐	☐

Section II: Access to medicaments

Where do you get the medicaments?

☐ Pharmacy
☐ Medical center
☐ Doctor
☐ Supermarket
☐ Family or friends
☐ Another source, please specify______________________________________

If pharmacy: could you please tell me which pharmacy and where is located?

____

Section III: Preferred options for illness treatment in the family

In the event of illness, do you prefer:

☐ Use of home-made treatments (ointments, infusions, teas, soups, etc)
☐ Self medication (known medicaments)
☐ Ask for treatment in the pharmacy
☐ Prescribed treatment by the doctor
☐ No treatment at all

Sección IV: Medicaments consumption

How often do you take pain medicaments or analgesics (e.g. Ibuprofeno, sevedol, dolex, buscapina, etc)?

☐ Once per week ☐ Once per month ☐ Once in 3 months ☐ Once in 6 months
☐ Never

How often do you take cold and / or cough medicine ?
(eg. Noraver, Adorem, Sinutab, Doloex-sinus, Bisolvon, Mucosolvan, Noraver tos, etc)

☐ Once per week ☐ Once per month ☐ Once in 3 months ☐ Once in 6 months
☐ Never

CHRONIC MEDICAL CONDITIONS
Are you or any family member under permanent medication?

☐ Yes ☐ No

Which analgesics and/or cold - cough medicine do you normally take?

Medicament	Brand	Active ingredients	Compound concentration	Intake doses per event

If yes, which medicaments are you taking?(Suggest the person to show you the packages of the products)

Medicament	Brand	Active ingredients	Compound concentration	Intake doses			
				Daily	Weekly	Monthly	Other

SECTION V: Disposal of medicaments

Where do you dispose the medicaments when they are not in use any more or when they have expired?

☐ In the container "blue point" for expired or non-used medicaments
☐ In the normal waste
☐ Through the toilet
☐ Bring them to the pharmacy
☐ Another, please specify: _____

SECTION VI: Consumption of hormones in contraceptive methods and hormone replacement treatment - The next questions are only for women in reproductive age (15-45 years old) –

Do you currently use any contraceptive method?

☐ Yes ☐ No

If yes, which one?

☐ Condom
☐ Contraceptive pill
☐ Contraceptive patch
☐ Subdermal implant
☐ Injectable contraceptive
☐ Intrauterine devices

☐ Other, please especify: _____

In case of use of any hormonal contraceptive method (contraceptive pill, contraceptive patch, subdermal implant, etc)

Medicament	Brand	Compounds	Compound concentration	Intake doses			
				Daily	Weekly	Monthly	Other

- The next questions are only for women in menopause -

Are you under hormonal substitution treatment (HST)?

☐ Yes ☐ No

In case of being under treatment, could you tell me which medicament are you taking?

Medicament	Brand	Compounds	Compound concentration	Intake doses			
				Daily	Weekly	Monthly	Other

Appendix 2.

List of most used pharmaceuticals in a population group in Bogotá, Colombia

Therapeutic use	**Compound**
Analgesic, antiinflammatory	Diclofenac
Analgesic, antipyretic	Acetaminophen
Analgesic, antipyretic, antiinflammatory, antiplatelet	Acetylsalicylic acid
Analgesic, antipyretic, antiinflammatory	Meloxicam
Antiasthmatic (Glucocorticoid steroid)	Beclometasone dipropionat
Antibodies inhibiting - anti-inflammatory	Adalimumab
Anticoagulant	Apixaban
Anticonvulsant	Phenytoin sodium
	Carbamazepine
Antidepressant	Fluoxetine
	Escitalopram oxalate
Antidiabetic	Human insulin
	Metformin
	Sitagliptin
	Glibenclamide
Antihyperlipidemic agent	Lovastatin
Anti-hypertensive	
-Angiotensin II receptor antagonists	Losartan
	Valsartan
	Irbesartan
-Angiotensin-converting-enzyme inhibitor	Enalapril
	Perindopril
-Beta blocker	Metoprolol tartrate
	Propanolol
-Calcium channel blocker	Verapamil
	Nifedipine
	Enalapril
	Flunarizine
-Diuretic	Hydrochlorothiazide
	Bisoprolol fumarate
	Furosemide
-Vasodilator	Clonidine
Antihyperuricemic agent	Colchicine

	Allopurinol
Antipsychotic	Olanzapine
Anxiolityc, anticonvulsant	Clonazepam
Gastric secretory inhibitor	Omeprazole
Gastrointestinal tract motility regulator	Treimebutine
Muscarinic receptor agonist	Pilocarpine chlorhidrate
Muscle relaxant	Methocarbamol
Opiate (sedative)	Codeine
Thyroid hormone	Levothyroxine

Appendix 3.

Protocol of extraction: pharmaceuticals in plant material (cereals)

Developed by the Zentrallabor Chemische Analytic Technische Universität Hamburg-Harburg (TUHH)

Principle: Solid-liquid extraction from milled cereals

Solid phase extraction, extract cleaning and fortification compounds

Chemicals:

- MeOH , 100% LiChrosolv and hypergrade
- MeOH with 0.1% Acetic acid
- Milli Q water
- Milli Q water with 0.1% Acetic acid
- Acetic acid 100%
- Di-sodiumhydrogenphosphate -2-hydrate (Na2HPO4)
- Citric acid
- Boric acid
- NaOH 20%
- Titriplex III (Ethylene diamine tetra acetic acid) EDTA
- Nitrogen gas

Equipment:

- Mill
- Ultrasonic bath
- Shaker
- Cetrifuge
- Biofuge
- Vacuum manifold
- Rotary evaporator

Material:

- Glassware: beakers, flasks, cylinders, 1L glass bottles (4).
- Pasteur pipettes /pears
- 50 ml centrifugation tubes
- SPE cartridges Bond Elute NEXUS 200 mg 6ml Angilent Technologies
- 50 ml round bottom flask
- 2 ml flasks
- Autosampler vials (with septum)

Preparation of sodium borate buffer (0.4, pH 9.5)

Weight 2.47 g Boric acid in a 100 ml beaker
Dissolve with 50 ml Milli Q water, and place in the stirrer at low temperature.
After dissolved, leave it to cool to room temperature and adjust the pH by adding 20% NaOH, filled up to 90 ml.
Measure the pH and fill up to 100 ml in a graduated flask.
IMPORTANT: 2.47g per 100 ml. The amount depends on the number of samples to extract.

Preparation of EDTA / Citric acid buffer solution (pH 4.1)

Solution 1: citric acid solution 0.1 mol/L
Weight 21.014 g of citric acid monohydrate and dissolve in 1 L Milli Q water
Solution 2: Di-sodiumhydrogenphosphate-2-hydrate 0.2 mol/L
Weight 35.6 g Di-sodiumhydrogenphosphate-2-hydrate or 28.4 g Di-sodiumhydrogenphosphate, and dissolve in 1 L Milli Q water

Final Solution:

Mix 620 ml of solution 1 with 380 ml of solution 2, and add 37.244 g of EDTA (0.1 mol/L).
Preparation MeOH/citric EDTA buffer solution (10/90 v/v)
Preparation of MeOH /boric acid buffer solution (10/90 v/v)
100 ml MeOH
900 ml boric acid
Preparation MeOH + 0.1% acetic acid
990 ml MeOH
10 ml acetic acid

Process

1. Samples preparation: samples are finely milled

2. Solid-liquid phase extraction
3. Weight two repetitions of 5g each sample
4. Place each sample in a 50 ml centrifugation glass tube and add 20 ml of MeOH/citric EDTA buffer solution (10/90 v/v). Mix by hand until homogeneous.
5. Place the tubes in the ultrasonic bath during 5 min.
6. Bring the tubes to the shaker and place them horizontally. Shake at 225 (rpm) for 60 min.
7. Centrifuge the tubes during 10 min at 6000 rpm
8. Solid phase extraction
9. Transfer the extract (supernatant) with a Pasteur pipette to the SPE cartridge and leave it to drain slowly (30-40 drops /min)
10. When the SPE is dry, leave it to blow dry under nitrogen gas for 30 min.
11. For compounds elution from the SPE add 10 ml of MeOH and then 10 ml of MeOH + 0.1% acetic acid. Transfer the extract into a 50 ml round bottom flask
12. Blow dry the SPE under nitrogen to renew. Repeat extraction process with residues in centrifuge tube:
13. Add 20 ml of MeOH /boric acid buffer solution (10/90) to the residues and mix thoroughly by hand.
14. Place for 5 min in the ultrasonic bath. After that place the tubes horizontally in the shaker for 30 min. Centrifuge at the same conditions as in 2.5.
15. Transfer supernatant with a Pasteur pipette to the same SPE column and slowly leave it to drain.
16. Leave SPE column to blow dry the under nitrogen gas for about 30 min.
17. Elute the compounds as in 3.3 in the same round bottom flask

Sample extract

- Leave the extract to evaporate in the rotary evaporator until more or less 1 ml extract remains
- Transfer the extract sample to a 2 ml flask and fill with MeOH up to the mark
- If the extract is too turbid, preserve the sample overnight in the fridge and then transfer the extract very carefully without mixing the sediments again to an EPI. Place the EPI in a Biofuge for 10 min at 13000 rpm.
- Collect the supernatant into autosampler vials for the analysis.