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Sanna Jaatinen

Characterization and Potential Use of Source-Separated Urine

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Characterization and Potential Use of Source-Separated Urine

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Supervisor: Professor Jukka Rintala
Department of Chemistry and Bioengineering
Tampere University of Technology
Tampere, Finland

Instructor: Professor Tuula Tuhkanen
Department of Biological and Environmental Science
University of Jyväskylä
Jyväskylä, Finland

Pre-examiners: Professor (emeritus) Leif Kronberg
Laboratory of Organic Chemistry
Åbo Akademi University
Turku, Finland

Dr.ir. Mariska Ronteltap
Senior Lecturer in Sanitary Engineering
Environmental Engineering and Water Technology Department
UNESCO-IHE Institute for Water Education
The Netherlands

Opponent: Dr. Lucía Hernández Leal
Scientific project manager
Wetsus, European Centre of Excellence for Sustainable Water Technology
The Netherlands

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Abstract

Human urine is an abundant source of the main nutrients (N, P, and K), while in the modern age it may contain traces of pharmaceutical and hormonal compounds. In many parts of the world, urine is collected into sewers, mixed with other wastewaters and treated at centralized wastewater treatment plants. Recently, the source separation of urine has been considered as a way to promote nutrient recovery from households as concentrated streams which could promote its use as fertilizer. The presence of pharmaceuticals and estrogens in urine has raised questions regarding its safe agricultural use, thus leading to a need to monitor the presence of pharmaceutically active compounds in urine. As a minimum, six-month storage before agricultural use is recommended for urine for hygienic reasons. The objective of this thesis was to develop and facilitate the use of source-separated urine by making the characterization of urine easier and faster, to gain more understanding regarding pharmaceutical and estrogenic behavior during urine storage, and to evaluate the suitability of human urine in microalgal cultivations as a nutrient source.

An analytical liquid chromatographic method, which had the advantage of simultaneous analysis of eight pharmaceuticals in a short six-minute analysis time, was developed as no such method previously existed (**Paper I**). The method was validated, proven repeatable, and the obtained pharmaceutical recoveries were acceptable (81.6–109.2%). The limit of detection for the pharmaceuticals in urine was 39–1 610 µg/L. In addition, a previously developed bioluminescent yeast cell biosensor *Saccharomyces cerevisiae* (BMAEREluc/ERα) was applied on urine samples, as prior to this thesis no information was available on the use of yeast biosensor in estrogenic activity assessment from source-separated human urine (**Paper II**). The biosensor produced repeatable results in the estrogenic activity testing of fresh and stored human urine with the limit of detection corresponding to 0.28–35 µg/L of estrogens. To enhance the signal, incubation with β-glucuronidase enzyme was used. The biosensor gave a cumulative signal for estrogenic activity (estrogens and estrogen-like compounds), thus enabling the assessment of overall estrogenic activity during urine storage.

The method presented in **Paper I** was subsequently used in monitoring spiked pharmaceutical concentrations in urine during six-month storage (**Paper III**). Each pharmaceutical (three antivirals and four antibiotic compounds) was tested in laboratory storage both individually and in therapeutic groups, as well as in therapeutic groups either with feces or urease inhibitor amendment. During storage, the overall concentration reductions of <1% to >99% were detected, and in assays with amendments, concentrations reductions remained <50%, except for rifampicin (>99%). Four of the pharmaceuticals had reduced concentrations after a six-month storage, suggesting biological or chemical degradation and/or precipitation of the compounds.

Human urine was tested as nutrient source for biomass production from microalga *Chlorella vulgaris* (**Paper IV**). Biomass yield in diluted urine was comparable with growth in artificial growth medium and urine could be utilized up to 1:25-dilutions without inhibition to algal growth. The highest biomass production (0.6 g/L) was achieved in 1:100-diluted urine. *C. vulgaris* used 32.5–78.7% of N and 35.1–99.0% of P available in urine. At the beginning of cultivation, the majority of the biomass consisted of algal cells, while towards the end the share of living algal cells decreased, indicating accumulation of bacteria and algal cell debris.

In conclusion, the results obtained in this thesis indicated that the pharmaceutical concentrations did not reduce enough in order to safely use urine. The yeast biosensor demonstrated that during storage, the estrogenic activity changes most likely due to bacterial enzyme activity, but some activity is still present after five months. The urine is a viable nutrient source for microalgal biomass production, thus having potential for sustainable use and recycling of nutrients.

Tiivistelmä

Ihmisen virtsa sisältää runsaasti pääravinteita (N, P ja K), mutta nykyaikana se voi myös sisältää jäämiä lääkeaineista ja hormoniyhdisteistä. Suuressa osassa maailmaa virtsa kerätään viemäreihin, sekoitetaan muiden kotitalous- ja teollisuusjätevesien kanssa ja käsitellään jätevedenpuhdistamoilla. Virtsan erilliskeräys kotitalouksissa on kiinnostava vaihtoehto tälle käytännölle, sillä tällöin ravinteiden kierrätystä voidaan edistää ja näin edesauttaa virtsan lannoitekäyttöä. Virtsan sisältämät lääkeaineet ja hormonit ovat herättäneet kysymyksiä turvallisesta lannoitekäytöstä, jonka vuoksi on tarve seurata ja tutkia erittyvien aktiivisten lääkeaineiden esiintymistä ja pitoisuuksia virtsassa. Virtsan hygienisoimiseksi ennen lannoitekäyttöä suositellaan minimissään kuuden kuukauden varastointia. Tämän väitöstyön tarkoituksena oli kehittää ja edistää erilliskerätyn virtsan käyttöä tekemällä virtsan sisältämien ainesosien tutkimisesta nopeampaa ja helpompaa, lisätä tietoa lääkkeiden ja hormonien käyttäytymisestä virtsan varastoinnin aikana, sekä arvioida virtsan soveltuvuutta halpana ja uusiutuvana ravinnelähteenä mikrolevien kasvatukseen.

Työssä kehitettiin uusi nestekromatografinen menetelmä kahdeksan lääkeaineen samanaikaiseen tutkimiseen kuuden minuutin analyysiajassa (**Julkaisu I**). Menetelmä validoitiin, se oli toistettava ja lääkeaineiden saannot olivat hyväksyttäviä (81,6–109,2 %). Lääkeaineiden määritysraja virtsassa oli 39–1610 µg/L. Lisäksi, aiemmin kehitettyä bioluminesoivaa hiivasolubiosensoria *Saccharomyces cerevisiae* (BMAEREluc/ERα) käytettiin virtsan estrogeeniaktiivisuuden tutkimiseen (**Julkaisu II**). Biosensori tuotti vertailukelpoiset tulokset tuoreen ja seisotetun virtsan estrogeeniaktiivisuusmittauksissa määritysrajan vastatessa 0,28–35 $\mu g_{estrogeeneja}$/L. Näytteiden signaalin vahvistamiseksi suoritettiin inkubointi β-glukuronidaasi-entsyymillä. Biosensori tuotti kumulatiivisen estrogeeniaktiivisuussignaalin (estrogeenit ja estrogeenien kaltaiset yhdisteet), joka mahdollistaa kokonaisestrogeeni-aktiivisuuden mittaamisen virtsan varastoinnin aikana.

Julkaisussa I esitettyä menetelmää käytettiin virtsaan lisättyjen lääkeaineiden pitoisuuksien tutkimiseen kuuden kuukauden varastoinnin aikana (**Julkaisu III**). Jokaista lääkeainetta (kolme viruslääkettä ja neljä antibioottiyhdistettä) tutkittiin yksittäin, terapeuttisissa ryhmissä sekä terapeuttisissa ryhmissä, joihin oli lisätty ulostetta tai ureaasi-inhibiittoria. Pitoisuudet vähenivät kaikissa kokeissa varastoinnin aikana <1–99 %, ja kokeissa, joissa käytettiin ulostetta tai ureaasi-inhibiittoria, pitoisuuksien vähenemät jäivät alle 50 % (paitsi rifampisiinilla yli 99 %). Pitoisuuksien selvät vähenemät neljällä yhdisteellä osoittivat biologista tai kemiallista hajoamista ja/tai saostumista.

Ihmisvirtsaa käytettiin ravinnelähteenä *Chlorella vulgaris* –mikrolevän biomassatuotannossa (**Julkaisu IV**). Biomassan saanto laimennetussa virtsassa oli vertailukelpoinen biomassatuotantoon keinotekoisessa kasvumediassa kasvatetun levän kanssa, ja virtsaa voitiin käyttää jopa 1:25-laimennettuna ilman levän kasvun inhiboitumista. Korkein biomassasaanto (0,6 g/L) saavutettiin 1:100-laimennetulla virtsalla. *C. vulgaris* käytti virtsassa olevasta typestä 32,5–78,7 % ja fosforista 35,1–99,0 %. Kasvatuksen alussa suurin osa biomassasta koostui leväsoluista, kun taas loppua kohden elävien leväsolujen osuus pieneni osoittaen bakteerien ja kuolleiden leväsolujen kertymistä kasvatukseen.

Tässä työssä saadut tulokset osoittivat, että lääkeaineiden pitoisuudet eivät alene varastoinnin aikana tarpeeksi, jotta virtsaa voisi turvallisesti käyttää. Hiivabiosensori osoitti, että varastoinnin aikana estrogeeniaktiivisuus virtsassa muuttuu todennäköisimmin bakteerien entsyymitoiminnan vuoksi, mutta aktiivisuutta on vielä jäljellä viiden kuukauden varastoinnin jälkeen. Ihmisen virtsa on myös käyttökelpoinen ravinnelähde mikroleväbiomassan tuotannossa, mikä edesauttaa ravinteiden kestävää käyttöä ja kierrätystä.

Preface

This thesis is based on the work carried out in the Department of Chemistry and Bioengineering, Tampere University of Technology, Finland. The Tampere University of Technology Doctoral Programme in Engineering and Natural Sciences, Finnish Doctoral Programme in Environmental Sciences and Technology (EnSTe), Maa- ja vesitekniikan tuki ry, Emil Aaltonen Foundation, and Tampereen kaupungin Tiederahasto are gratefully acknowledged for their financial support for the completion of this thesis. I'm grateful for Emeritus professor Leif Kronberg (Åbo Akademi University) and Dr.ir. Mariska Ronteltap (UNESCO-IHE Institute for Water Education) pre-viewing this thesis and giving valuable comments.

I want to give my thanks to Professors Jukka Rintala and Matti Karp from Tampere University of Technology for giving me guidance and support throughout this journey. My thanks belong also to Professor Tuula Tuhkanen, from University of Jyväskylä, to whom I owe gratitude for helping me set up this journey towards a doctoral degree and leading me to the fascinating world of sustainable sanitation and pharmaceuticals. Without the three of you I would not be here now.

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Last but not least, I want to thank my friends, my husband Toni and my mother Ulla, for being supportive throughout these years of thesis preparation. Sometimes, when motivation was low, you gave me the kick in the backside I needed. I also want to thank our german shepherd Redi for not eating all my notes and destroying my nerves, although it was sometimes very close. This has been a long journey, including a lot of bumps and obstacles on the road. If someone would have told me beforehand how difficult and stressing thesis writing would be, I still would have done it!

Tampere, June 2016

Sanna Jaatinen
Sanna Jaatinen

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List of Original Publications

This thesis is based on the following original publications which are referred to in this thesis by the roman numerals **I-IV**. The publications are reproduced with kind permissions from the publishers.

I Pynnönen, S.T., Tuhkanen, T.A. 2014. Simultaneous detection of three antiviral and four antibiotic compounds in source-separated urine with liquid chromatography. *Journal of Separation Science* 37(3), pp. 219–227. doi: 10.1002/jssc.201300492

II Jaatinen, S., Kivistö, A., Palmroth, M.R.T., Karp, M. 2016. Effect of source-separated urine storage on estrogenic activity detected using bioluminescent yeast *Saccharomyces cerevisiae*. *Environmental Technology*. doi: 10.1080/09593330.2016.1144797

III Jaatinen, S.T., Palmroth, M.R.T., Rintala, J.A., Tuhkanen, T.A. 2016. The effect of urine storage on antiviral and antibiotic compounds in the liquid phase of source-separated urine. *Environmental Technology.* doi: 10.1080/09593330.2016.1144799

IV Jaatinen, S., Lakaniemi, A-M., Rintala, J. 2016. Use of diluted urine for cultivation of *Chlorella vulgaris*. *Environmental Technology* 37(9), pp. 1159–1170. doi: 10.1080/09593330.2015.1105300

Author's Contribution

Paper I: Sanna Jaatinen (née Pynnönen) wrote the paper and is the corresponding author. She planned the experiment, performed the experimental work, and interpreted the results.

Paper II: Sanna Jaatinen wrote the paper, performed the experimental work, interpreted the results, and is the corresponding author. Dr. Anniina Virtanen (née Kivistö) assisted in planning the experiment and Dr. Marja Palmroth in interpretation of the results.

Paper III: Sanna Jaatinen wrote the paper and is the corresponding author. She planned the experiment, performed the experimental work, and interpreted the results. Dr. Marja Palmroth assisted in interpretation of the results.

Paper IV: Sanna Jaatinen wrote the paper, performed the experimental work, and is the corresponding author. Dr. Aino-Maija Lakaniemi assisted in planning the cultivation experiment and in interpretation of the results.

I wrote the first draft of all the papers and finalized them with my coauthors and supervisors. The experimental work was carried out under the supervision of Prof. Matti Karp (**Paper II**), Dr. Aino-Maija Lakaniemi (**Paper IV**), Dr. Marja Palmroth (**Papers II** and **III**), Prof. Jukka Rintala (**Papers III** and **IV**), and Prof. Tuula Tuhkanen (**Papers I** and **III**).

Abbreviations

3TC	Lamivudine
AAS	Atomic adsorption spectrophotometer
ACN	Acetonitrile
CBZ	Carbamazepine
CIP	Ciprofloxacin
C:N	Carbon to nitrogen ratio
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical oxygen demand
cfu	Colony forming unit
DOC	Dissolved organic carbon
E_1	Estrone
E_2	17β-estradiol
EE_2	17α-ethinylestradiol
E_3	Estriol
EDC	Endocrine disrupting compound
ER	Estrogen receptor
EtOH	Ethanol
EU	European Union
FI	Fold induction
FL	Fluorescence
GC	Gas chromatography
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
LC-(ESI-)MS/MS	Liquid chromatography- (electrospray ionization) -tandem mass spectrometry
LOD	Limit of detection
$\log K_{ow}$	Logarithm of octanol-water partition coefficient
LOQ	Limit of quantification
MeOH	Methanol
MFC	Microbial fuel cell
MS	Mass spectrometer
nBPT	N-(n-butyl) thiophosphoric triamide
NH_4-N	Ammonium-nitrogen
N_{org}	Organic nitrogen
N_{tot}	Total nitrogen
NVP	Nevirapine
OD_{600}	Optical density @600 nm
PCB	Polychlorinated biphenyl
pK_a	Acid dissociation constant

ppb	parts per billion, 10^{-9}
P_{tot}	Total phosphorus
RMP	Rifampicin
SAG	Culture Collection of Algae
SFS	Finnish Standards Association
SMX	Sulfamethoxazole
SPE	Solid phase extraction
TOC	Total organic carbon
TRI	Trimethoprim
TUT	Tampere University of Technology
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen
ZDV	Zidovudine

1 Introduction

Humans produce urine on a daily basis, as it has been from the beginning of times, and the composition and quantity of urine vary by the person and diet. There are currently over 7.3 billion people in the world and each and every one of them produces approximately 1.2 L of urine per day, containing 11.5 gN/p/d and 1.2 gP/p/d (Kujala-Räty *et al.* 2008). Thus, the amount of urine in the world reaches 8.85 billion m^3/d with $84 \cdot 10^3$ tons N and $8.8 \cdot 10^3$ tons P. As a reference, the world inorganic fertilizer (as NPK) consumption in years 2005–2007 was $166 \cdot 10^6$ tons with division of 57% N, 25% P, and 18% K (FAO 2012). The estimation for fertilizer consumption in year 2050 has been projected as $263 \cdot 10^6$ tons (FAO 2012).

Human urine contributes approximately 80–90%, 50–65%, and 50–80% of the N, P and K, respectively, arriving at typical centralized wastewater treatment plants (WWTPs), while it is only 0.4–1.0% of the volume (Höglund *et al.* 2002, Heinonen-Tanski and van Wijk-Sijbesma 2005, Pronk *et al*. 2006; Vinnerås *et al.* 2008, Winker *et al.* 2008a, Jana *et al.* 2012b). Centralized WWTPs have been developed from stormwater sewers which were originally constructed to prevent flooding of urban areas and further used to transport human excreta in response to the need to ensure hygiene and health in urban areas (Wilsenach and van Loosdrecht 2004). Along the development, the flush toilet was introduced and it became of a symbol for cleanliness (Medilanski *et al.* 2006). Nowadays in many cases, domestic wastewaters (toilet, kitchen, personal hygiene) are mixed together in the sewers with rainwater and infiltration water, in addition to with e.g. industrial and hospital wastewaters (Kümmerer 2001, Medilanski *et al.* 2006), and are treated in a centralized WWTP (Remy and Jekel 2012). Meanwhile, the centralized system has received criticism, as it is not considered sustainable (Wilsenach and van Loosdrecht 2004). Therefore, other systems, such as the source separation of urine, have been shown to have environmental benefits in environmental life-cycle studies (Jönsson 2002, Maurer *et al.* 2003, Spångber *et al.* 2014). Separating urine from other domestic wastewaters can reduce the organic and nutrient load arriving at WWTPs.

Human excreta, including urine, could be considered as a resource rather than a waste as urine contains essential nutrients, already in a form available to plants (Sundin *et al.* 1999, Lind *et al.* 2001, Golder *et al.* 2007, Yang *et al.* 2015). Source separation and use of human excreta

as a fertilizer is even claimed to be more energy-efficient and have less impact on global warming than the enhanced reduction of nitrogen and phosphorus at a WWTP, complemented with the use of chemical fertilizers (Spångberg *et al.* 2014). However, urine may contain microorganisms, pharmaceuticals, and hormones and thus, their fate and removal during urine storage and re-use need to be addressed before urine may be regarded as safe. Therefore, separating urine from other wastewaters could help to lessen the pharmaceutical and hormonal burden of both WWTPs and the environment, in addition to recycling nutrients in a more sustainable way. As pharmaceuticals and hormones enter the environment, they pose a threat to e.g. aquatic life by increasing the occurrence of hermaphroditic fish near WWTPs (Larsson *et al.* 1999), by re-entering the water cycle (Kujawa-Roeleveld *et al.* 2008), or by accumulating in soils and plants (Pronk *et al.* 2006), and concerns have risen about the potential effects of these compounds on aquatic organisms and human health during long-term, continuous exposure (Escher *et al.* 2006). The presence of pharmaceuticals and estrogenic hormones in urine-based fertilizer products is also a matter of concern.

Pharmaceuticals have been detected from municipal WWTPs with varying daily loads (Sim *et al.* 2011). As pharmaceuticals, hormones, and hormone-like ingredients cover a wide variety of compounds, detecting them in collected urine in order to monitor the effectiveness of urine treatment techniques has been gaining interest, as have their concentrations in the environment and in urine-based fertilizer products (Ronteltap *et al.* 2007). A necessity has risen to develop simple and rapid analytical techniques to facilitate the re-use of urine. Resulting from a faster urine analysis, the utilization of urine in fertilization applications becomes simpler since the presence of harmful substances is rapidly discovered. Thus, the suitability of urine in microalgal cultivation and biomass production, for instance, can be more simply assessed.

The objective of this thesis was to develop and facilitate the use of source-separated urine by developing and assessing the feasibility of analytical tools in urine monitoring, by evaluating the behavior of pharmaceuticals during urine storage, and by assessing the feasibility of urine as a nutrient source in microalgal cultivation and biomass production. In the following chapters, the composition and uses of human urine, as well as techniques used in source-separated urine treatment are discussed (**Chapters 2.1.1-2.1.4)**. Related to pharmaceutical excretion, a short summary on pharmaceutical metabolism in human body is given (**Chapter 2.2.1**), and the fate of pharmaceutical compounds in urine treatment processes and at WWTPs is discussed (**Chapter 2.2.2**). As pharmaceuticals and estrogens have environmental relevance and need to be monitored from environmental and urine samples, an insight on analytical techniques for their detection is given (**Chapter 2.3**). Afterwards, the aims of the thesis are presented (**Chapter 3**) and the materials and methods used are shortly described (**Chapter 4**). Subsequently, the results and discussion related to analytical development, pharmaceutical and estrogenic monitoring, as well as results from urine storage and its effects on pharmaceutical behavior in urine, and a description of the algal cultivation experiments conducted in urine are given (**Chapter 5**). Thereafter, conclusions, based on findings presented in the thesis, are summarized (**Chapter 6**).

2 Background

2.1 Human Urine

2.1.1 Composition of Human Urine

Human urine is a complex water solution, containing nutrients as diluted compounds. Main compounds are sodium chloride (NaCl, 9-16 g/d) and urea [$CO(NH_2)_2$] (25 g/d), which is the major source of total nitrogen (80–90%) in fresh urine (up to 9 gN/L). Also potassium (K, 50–80%), calcium (Ca), sulphate (SO_4) and phosphorus (approximately 0.7 gP/L, 50–65%) are present (**Table 2.1**). Urine contains an excess of ammonium relative to phosphate, but it is deficient in magnesium. Phosphorus is available as phosphates ($H_2PO_4^-$ or HPO_4^{2-}) and potassium as an ionic component (K^+). Urine contains organic compounds which are breakdown products of biomolecules and food, such as hydroxyindoles, ethyl mercaptan and other sulphides, phenols and cresols, as well as substituted benzoic and phenylacetic acids (Escher *et al.* 2005 end references therein). Urine also contains uric acid, chlorides, oxalates, and small amounts of minerals, vitamins, hormones, amino acids, and enzymes. (Harper *et al.* 1979, Lind *et al.* 2001, Heinonen-Tanski and van Wijk-Sijbesma 2005, Ganrot *et al.* 2007a, Winker *et al.* 2009). The nutrient and chemical composition of urine, reported in the literature, is presented in **Table 2.2**.

The quantity and composition of urine depend on age, water intake, diet, and external temperature. Normally, an adult produces 0.6 to 2.5 L of urine daily (average being 1.2 L). Urine is normally acid, with pH ranging from 4.7 to 8.0 (average pH 6.0). Normal color of urine is pale yellow or amber, the determining pigment being urochrome, but color varies with its concentration; also pharmaceuticals can cause colored urine. (Harper *et al.* 1979). Each year, one person produces approximately 500 kg of urine (Heinonen-Tanski and van Wijk-Sijbesma 2005) which includes 2.5–5.7 kgN, 0.3–1.0 kgP and 0.1–1.2 kgK per person per year (Kirchmann and Pettersson 1995, Heinonen-Tanski and van Wijk-Sijbesma 2005, Mihelcic *et al.* 2011).

TABLE 2.1. Composition of urine (modified from Harper *et al.* 1979).

Urine property/component	Unit	In urine
specific gravity	kg/L	1.003–1.030
pH	-	4.7–8.0
volume	mL	600–2500
acidity (as 0.1 N NaOH)	mL	250–700
total solids	g/L	30–70
Inorganic constituents (per 24 h)		
chlorides (NaCl)	g	9–16
Sodium (varies with intake)	g	4
Phosphorous	g	2–2.5
Potassium (varies with intake)	g	2
Sulfur (as SO_3)	g	0.7–3.5
Calcium	g	0.1–0.2
Magnesium	g	0.05–0.2
Iodine	µg	50–250
Organic constituents (per 24 h)		
nitrogenous (total)	g	25–35
urea (varies with diet)	g	25–30
creatinine	g	1–1.8
ammonia	g	0.3–1
uric acid	g	0.5–0.8
undetermined N (amino acids etc.)	gN-eq	0.5
protein, e.g. albumin	g	0–0.2
creatine	mg	60–150
Other organic constituents (per 24 h)		
hippuric acid	g	0.1–1
oxalic acid	mg	15–20
indican	mg	4–20
coproporphyrins	µg	60–280
purine bases	mg	10
ketone bodies	mg	3–15
allatoin	mg	30
phenols (total)	g	0.2–0.5
ascorbic acid	mg	15–50

Ureases (urea amidohydrolases) are a group of enzymes widely synthesized in nature by plants, bacteria, fungi, algae, and invertebrates; they also occur in soils as a soil enzyme (Krajewska 2009). When urine is left standing, urea is hydrolyzed to ammonia and carbamate by urease, and the latter compound decomposes spontaneously to carbonic acid and another molecule of ammonia; the process is called ureolysis (**Eq. 1.1**, Udert *et al.* 2003). Without urease, urea is a very stable compound, with a half-life time of 3.6 years at 38°C (Hotta and Funamizu 2008 and references therein).

$$NH_2(CO)NH_2 + 2 \cdot H_2O \rightarrow NH_3 + NH_4^+ + HCO_3^- \qquad (\mathbf{1.1})$$

Due to the ammonia release, pH increases (Udert *et al.* 2003). During ureolysis precipitates are formed, of which struvite ($MgNH_4PO_4 \cdot 6H_2O$) is the most commonly studied and dominates precipitation at high pH (Udert *et al.* 2003). Struvite is an orthophosphate, which contains

magnesium, ammonium, and phosphate in equal molar concentrations (Ronteltap *et al.* 2007). Ureolysis thus leads to phosphorus and magnesium precipitation.

TABLE 2.2. Nutrients and chemical composition of human urine reported in the literature.

Composition	Unit	Stored[a]	Stored[b]	Stored, undiluted	Fresh	Fresh	Stored	Fresh
N_{tot}	g/L	1.795	2.610	9.2	8.83	-	-	-
NH_4-N	g/L	1.117	1.726	-	-	0.254	1.720	0.438
NH_3aq-N	g/L	0.574	0.773	8.1	0.463	-	-	-
urea	g/L	-	-	-	-	5.810	0.0073	4.450
amino acid –N	g/L	0.104	0.110	-	-	-	-	-
NO_3-N	µg/L	45	45	-	-	-	-	-
NO_2-N	µg/L	10	20	0	-	-	-	-
pH	-	8.96	8.90	9.1	6.2	7.2	9.0	5.6
conductivity	mS/cm	13.4	19.0	-	-	-	-	22.6
redox potential	mV	-90	+236	-	-	-	-	-
COD	g/L	-	-	-	-	8.150	1.650	7.660
Macronutrients								
P	g/L	0.210	0.200	0.540	0.8-2.0	0.367	0.076	0.388
K	g/L	0.875	1.150	2.2	2.737	2.170	0.770	1.870
SO_4-S	g/L	0.225	0.175	0.505	1.315	0.748	0.292	0.878
Na	g/L	0.982	0.938	2.6	3.45	2.670	0.837	3.240
Cl	g/L	2.500	2.235	3.8	4.97	3.830	1.400	6.620
Micronutrients								
Ca	mg/L	15.75	13.34	0	0.233	129	28	89.2
Mg	mg/L	1.63	1.5	0	0.119	77	1	45.4
Mn	mg/L	0	0	-	0.0019	-	-	-
Fe	mg/L	0.205	0.165	-	-	-	-	-
B	mg/L	0.435	0.440	-	0.097	-	-	-
Al	mg/L	0.210	0.185	-	-	-	-	-
Reference		Kirchmann and Perttersson (1995)		Maurer *et al.* (2006)		Udert *et al.* (2003)		Etter *et al.* (2011)

Note: [a] stored 0–3 months; [b] stored 6 months; - data not reported

Some bacteria that inhabit the urinary tract excrete ureases that take part in ammonium formation and pH rise (Krajewska 2009). One example of these microorganisms is *Proteus mirabilis*, which is also responsible for producing urinary stones (Krajewska 2009), and blocking urinary tract catheters in hospitals and urine pipelines in urine diversion systems (Udert *et al.* 2003, Maurer *et al.* 2006) due to struvite precipitation. Ureases have an optimum pH range from pH 6 to pH 9, while also acid ureases exist (pH optimum 2–4.5) (Krajewska 2009).

Human urine may contain pathogens (e.g. *Salmonella typhi*, *S. paratyphi, Mycobacterium tuberculosis,* and *Leptospira interrogans*) and few enteric microorganisms, viruses (e.g. Cytomegalovirus), protozoa (e.g. Microsporidia), or helminth and parasitic eggs (e.g. *Schistosoma haematobium*) which can be emitted in urine (Höglund 2001, Höglund *et al.* 2002, Heinonen-Tanski and van Wijk-Sijbesma 2005, Vinnerås *et al.* 2008). Urine has been shown to contain none or only low levels of the infectious human immunodeficiency virus (HIV) but does not appear to be an important source of virus transmission (Levy 1993). In urine, ammonia concentration of 40 mM, which can be formed during storage, has been established as an inactivation threshold for pathogenic microorganisms (Vinnerås *et al.* 2008). When in the

bladder, human urine is sterile (Höglund 2001). However, collected human urine is not totally sterile, as it contains fecal contamination which in source-separated urine is mainly due to misplaced feces: the average contamination is 9.1 mg_{feces}/L urine (Schönning *et al.* 2002) and can be measured by monitoring the concentration of fecal sterols (Sundin *et al.* 1999).

Urine also contains heavy metals in a ppb-range (Vinnerås *et al.* 2008, **Table 2.3**), while the differences in heavy metal concentrations vary depending on the time of storage and possible precipitation of metals. Urine may also contain pharmaceuticals, as approximately 60–70% of human pharmaceuticals and hormones are excreted in urine (Lienert *et al.* 2007), the topic of which will be discussed in more detail in **Chapter 2.2**. Urine may also contain traces of the chemicals that humans are exposed to on a daily basis, such as plastic softeners and flame retardants (Breithaupt 2014).

TABLE 2.3. Reported heavy metal contents of urine.

Heavy metal (µg/L)	**Urine, stored 3–6 months**	**Urine, hydrolyzed**
Cd	0.2	5.0
Cr	2.0–4.0	11.0
Co	1.0–12.0	13.8
Cu	155	88.4
Hg	0.44–0.55	-
Ni	15–227	8.1
Pb	2.0	27.2
Zn	70–110	-
Al	185–210	-
As	-	151
Reference	Kirchmann and Perttersson (1995)	Ronteltap *et al.* (2007)

Note: - data not found

2.1.2 Source Separation, Storage, and Treatment of Urine

Source separation basically means the separation and separate collection of urine from feces in the toilet, thus enabling the (re-)use of collected urine as such in different applications. Different techniques for source separation of urine have been proposed; e.g. urine diverting toilets (NoMix toilets, Medilanski *et al.* 2006) and waterless urinals, which are used to collect little diluted urine (Udert *et al.* 2003). NoMix toilets, depending on the model, use 0.2–0.8 L of flushing water (Udert *et al.* 2003) compared with the 2.5–4 L in the conventional double-flush toilets. Separating toilets typically consist of a bowl divided into two parts: the front bowl collects the urine and the rear bowl the feces (Schönning *et al.* 2002, Kvarnström *et al.* 2006, Larsen *et al.* 2009). After separation urine is collected in a storage tank in or near the household, and treated on-site or transported further to a centralized treatment unit (Borsuk *et al.* 2008, Larsen *et al.* 2009).

Source separation of urine has been studied in a larger scale for instance in a pilot village in Sweden (Kvarnström *et al.* 2006, Johansson *et al.* 2009), in office-scale in Germany (23 waterless urinals and 43 NoMix-toilets, Blume and Winker 2011; Schürmann *et al.* 2012), and

in a university in Australia (Abeysuriya *et al.* 2013). A larger field study conducted in the eThekwini Municipality in South Africa with 700 households (i.e. the VUNA project, Valorization of Urine Nutrients in Africa) has demonstrated the applicability of urine diverting dry toilets in collecting material for fertilizer production (Etter *et al.* 2014a, 2014b, Rhonton *et al.* 2014). A lot of information is available for different separation techniques and related issues, such as maintenance of toilets and user opinions (e.g. Johansson 2000, Kvarnström *et al.* 2006, Borsuk *et al.* 2008, Larsen *et al.* 2009, Lienert and Larsen 2009, Larsen *et al.* 2010, Blume and Winker 2011).

During urine storage, ureolysis (**Eq. 1.1**) causes ammonia losses due to volatilization. Pathogenic contamination and infections from enteric viruses present in urine are mainly dependent on the fecal cross-contamination and storage time as well as temperature. The recommended storage time at 20°C is at least six months, after which urine is considered safe to use as a fertilizer for any crop since pathogenic microorganisms have assumed to be dead and/or inactivated. (Höglund *et al.* 2002, WHO 2006, Winker *et al.* 2009). The enteric micro-organisms usually die off in two months' time, which is why shorter than six-month storage periods are commonly applied (Akpan-Idiok *et al.* 2012).

Different urine treatment techniques can be applied on the collected urine and are based on separation processes, e.g. membranes or precipitation, and elimination processes, e.g. oxidation or adsorption (Maurer *et al.* 2006), and have been discussed in the literature (**Table 2.4**) for e.g. hygienization, volume reduction, stabilization, and nutrient removal and recovery perspectives, and also for pharmaceutical removal (for a review, see Maurer *et al.* 2006, Tettenborn *et al.* 2007). The technologies exist and the separation process of nutrients and pharmaceuticals is relevant to the production of a urine-based fertilizer, whereas the pharmaceuticals must be eliminated for environmental pollution control (Maurer *et al.* 2006). Some of these techniques can be used in pharmaceutical concentration reduction, which will be discussed further in **Chapter 2.2**. Only urine storage has been investigated thoroughly enough for its ability to reduce the amount of pathogens in source-separated urine, while many of the other treatment steps will probably also have an effect on the hygienic properties (Maurer *et al.* 2006). For example, the nitrification/distillation process introduced by Udert *et al.* (2015) stabilizes nutrients by biological treatment and additionally improves the quality of the liquid fertilizer by inactivating pathogens and removing pharmaceuticals with post-nitrification distillation and advanced treatment, respectively (Bischel *et al.* 2015).

Some of these treatment techniques (ozonation, nanofiltration, electrodialysis, distillation) are high-tech processes and consume energy for e.g. heating, pressure provision, etc. (Gulyas *et al.* 2007, Tettenborn *et al.* 2007, Udert and Wächter 2012). Based on the comparison performed by Dodd *et al.* (2008), the energy requirements for urine pre-treatment may be lower than those required to achieve similar effluent quality via enhanced wastewater treatment, based on the energy savings derived from upstream resource recovery.

TABLE 2.4. Different urine treatment processes and their aims described in the literature.

Urine treatment process	Aim of treatment	Application scale	Reference
acidification	stabilization (prevention of precipitation, degradation and volatilization)	laboratory	Maurer *et al.* (2006)
adsorption	ammonia and phosphate removal from urine with e.g. zeolite	laboratory	Tettenborn *et al.* (2007)
crystallization	crystal formation, fertilizer product	laboratory	Tettenborn *et al.* (2007)
electrochemical oxidation on graphite	ammonia removal	laboratory	Udert *et al.* (2015) Zöllig *et al.* (2015)
electrodialysis	salt concentration, pharmaceutical concentration reduction	laboratory, pilot tests	Dodd *et al.* (2008). Escher *et al.* (2006) Pronk *et al.* (2007)
evaporation	volume reduction and concentration of nutrients, pharmaceutical concentration reduction	laboratory, pilot, 18 L of concentrate from 1 m^3 of urine	Tettenborn *et al.* (2007)
freeze-thaw	volume reduction	laboratory	Maurer *et al.* (2006)
ion exchange	nitrogen recovery	laboratory	Maurer *et al.* (2006)
microbial electrolysis cell	hydrogen production ammonium recovery	laboratory	Kuntke *et al.* (2014)
microbial fuel cells	energy production nitrogen recovery ammonium recovery nitrogen and phosphorus removal pharmaceutical removal	laboratory	Ieropoulos *et al.* (2013) Kuntke *et al.* (2012) Ledezma *et al.* (2015) Santoro *et al.* (2013a) Santoro *et al.* (2013b) Wang *et al.* (2015)
micro- and nanofiltration	turbidity removal, separation of nutrients from pharmaceuticals, pharmaceutical concentration reduction	laboratory	Pronk *et al.* (2006, 2007)
nitrification/distillation	nutrient recovery partial degradation of pharmaceuticals	laboratory, pilot tests	Bischel *et al.* (2015) Fumasoli *et al.* (2016) Udert *et al.* (2015) Udert and Wächter (2012)
ozonation	pharmaceutical concentration reduction, additional treatment	laboratory, pilot tests	Dodd *et al.* (2008) Escher *et al.* (2006) Tettenborn *et al.* (2007) Pronk *et al.* (2007)
precipitation	solid fertilizer, mainly struvite	pilot	Escher *et al.* (2006) Maurer *et al.* (2006) Ronteltap *et al.* (2007) Tettenborn *et al.* (2007)
reverse osmosis	volume reduction	laboratory	Maurer *et al.* (2006)
steam stripping	nitrogen recovery, pharmaceutical concentration reduction	laboratory, pilot plant, >2 m^3	Tettenborn *et al.* (2007)
storage	hygienization	pilot and technical	Maurer *et al.* (2006)
UV-radiation	pharmaceutical concentration reduction, additional treatment	laboratory	Tettenborn *et al.* (2007)

Note: UV - ultraviolet

In the future, urine treatment would most probably consist of a combination of treatment processes, e.g. by removing phosphate and ammonium by struvite precipitation followed by a biological process to eliminate organic pollutants and nitrogen (Maurer *et al.* 2006) either at the source or at centralized treatment units. One of the novel techniques include the use of microbial fuel cells (MFCs) to recover nitrogen while simultaneously producing electricity (Kuntke *et al.* 2012, Santoro *et al.* 2013); the objective being energy-positive wastewater treatment (Ledezma *et al.* 2015). MFCs can also be utilized in micropollutant (i.e. trace organic compounds) removal from wastewater (for a review, see Wang *et al.* (2015)).

2.1.3 Use of Human Urine

As urine is rich in nutrients, it has inspired research in the field of fertilizer use in different applications. According to the European Union (EU), the concept of *urine* as a fertilizer is unknown: only chemically pure urea is accepted in fertilizer use on agricultural fields and it is regarded as inorganic fertilizer (Regulation No 2003/2003). Regarding manure, instructions on its use are given in a Council regulation, but (human) urine is not involved (Regulation No 1069/2009). Legislation dealing with organic farming in the EU denies the use of human urine in agricultural fields (Council Regulation 834/2007). However, World Health Organization has introduced guidelines for safe re-use of human excreta (WHO 2006); next, a short summary of source-separated urine as a fertilizer is given.

Urine as a Fertilizer in Agri- and Aquaculture

As discussed in the previous section, human urine contains nutrients that are needed for plant growth, thus making it an appealing fertilizer alternative (Paruch 2012). Human urine has also been recognized as a potential aquacultural nutrient source.

Traditionally, urine as such has been and can be used in liquid form which can be applied with conventional equipment available at farms, or it can be precipitated as struvite which is a rather pure mineral (Winker *et al.* 2009). Struvite crystallizes when human urine is treated with magnesium (Mg^{2+}) (Maurer *et al.* 2006) and can then be used as a slow-release phosphatic fertilizer (nutrient becomes available gradually over time, Ronteltap *et al.* 2010) (Antonini *et al.* 2012). Human urine releases plant nutrients faster than sources such as livestock/avian excreta, green manure, compost manure, or similar (Akpan-Idiok *et al.* 2012). In developing countries, the use of human urine could be a solution as a low-cost, easily available and safe fertilizer (Jana *et al.* 2012a). Human urine has been used to fertilize edible plants (**Table 2.5**) with no considerable changes in taste and with better yields than with artificial fertilizers (Heinonen-Tanski *et al.* 2007, Pradhan *et al.* 2007, Tidåker *et al.* 2007, Pradhan *et al.* 2009, Pradhan *et al.* 2010, Akpan-Idiok *et al.* 2012).

The prevention of ammonia loss during storage and soil application of urine is an important aspect for efficient fertilizer use (e.g. Kirchmann and Pettersson 1995, Watson *et al.* 2008). Indeed, urine infiltrates soil quite quickly and thereafter ammonia emissions end. Therefore, the application technique has to be adjusted to the high ammonia content of urine (i.e., close

to soil preferably in combination with soil incorporation) (Winker *et al.* 2009). By using a struvite fertilizer, ammonia volatilization is minimized compared with direct urine application into soil (Heinonen-Tanski and van Wijk-Sijbesma 2005, Watson *et al.* 2008).

Soil ureases (Krajewska 2009) rapidly hydrolyze urea, leading to ammonia emissions when urine is applied into the soil (Watson *et al.* 2008, Saggar *et al.* 2013, Singh *et al.* 2013). In order to prevent the loss of ammonia from urea during soil application, the use of urease inhibitors has been studied (Watson *et al.* 2008). The most promising urease inhibitor in different laboratory and field studies has been N-(n-butyl) thiophosphoric triamide (nBPT), a structural analogue of urea, which is effective in low concentrations, non-toxic, stable, and inexpensive (Watson *et al.* 2008, Saggar *et al.* 2013), as well as available commercially as e.g. Agrotain® (Zaman and Blennerhassett 2010, Singh *et al.* 2013) and StabilureN® (Agra Group 2015). Granular urea amended with nBPT reduced ammonia losses under laboratory conditions from 11% to 1.9% of urea-N applied and no evidence of nBPT efficiency decline was detected after repeated applications on the same soil over a three-year period (Watson *et al.* 1998).

Use of urine in aquaculture, such as in hydroponic cultivation of plants or growing zoo- and phytoplankton, is another way to potentially use the renewable nutrient source (**Table 2.5**). One of the issues associated with fresh urine use in aquaculture is the high concentration of ammonia and high pH which can be harmful to aquatic species (Jana *et al.* 2012a). Therefore, aeration of urine is usually used in urine-fed aquacultural systems as it transforms ammonia to nitrate more rapidly via nitrification (Jana *et al.* 2012a). Another advantage of aeration is the reduction of anaerobic pathogens which may be present in urine-fed culture systems (Jana *et al.* 2012a). However, the toxicity of urine at higher concentrations can pose risks to e.g. zooplankton survival, which is why urine needs to be highly diluted (Golder *et al.* 2007). It has been demonstrated that human urine can be used in aquaculturing of microalgae, zoo- and phytoplankton, and tomatoes (Adamsson 2000, Golder *et al.* 2007, Jana *et al.* 2012b), as well as fish and prawns (Jana *et al.* 2012a) and water spinach (Yang *et al.* 2015). In most cases, cultivation in diluted urine led to similar or higher yields than in nutrient solution or compared with other nutrient sources used (cow urine, vermi-compost etc., **Table 2.5**).

TABLE 2.5. Studies reported in the literature on ways to utilize human urine as a fertilizer.

Agricultural applications	**Scale of cultivation**	**Urine application**	**Main observation**	**Reference**
fertilization with fresh human urine				
cabbage	outdoor cultivation	180 kgN/ha	growth and biomass were slightly higher with urine than with a mineral fertilizer	Pradhan *et al.* (2007)
cucumbers	outdoor cultivation	233 kgN/ha(urine)[a] 34 kgN/ha (mineral)[a]	the cucumber yield with urine was similar or slightly better than the yield obtained with a mineral fertilizer	Heinonen-Tanski *et al.* (2007)
red beet	outdoor cultivation urine only, urine+ash	133 kgN/ha	urine and ash, and only urine fertilizer produced 1720 and 656 kg/ha more root biomass than mineral fertilizer, respectively	Pradhan *et al.* (2010)
wheat	life cycle analysis	-	-	Tidåker *et al.* (2007)
	climate chamber, 21 days, pot trials (freeze-concentrated urine, urine-equilibrated zeolite, urine-equilibrated activated carbon, struvite)	0.45-0.65 gN/kg[b]	the struvite/adsorbent mixtures showed better nutrient availability than struvite alone, but nutrients weren't enough for wheat growth during 21 days	Ganrot *et al.* (2007b)
okra	greenhouse and field	0, 45.8, 68.7 or 91.6 kgN/ha[c]	91.6 kgN/ha significantly increased the growth and yield relative to inorganic fertilizer; 68.7 kgN/ha had similar effect as inorganic fertilizer	Akpan-Idiok *et al.* (2012)
fertilization with stored human urine				
tomato	greenhouse experiment	135 kgN/ha	urine fertilization resulted in equal amounts of tomato fruits as mineral fertilizer and 4.2 times more fruits than non-fertilized plants	Pradhan *et al.* (2009)
fertilization with struvite				
maize, ryegrass	greenhouse pot experiment	0.17 gN/kg[d]	struvite fertilizers induced similar or significantly higher biomass yields than those generated by a commercial mineral fertilizer	Antonini *et al.* (2012)

TABLE 2.5. Continues.

Aquacultural applications *fresh and stored urine*	**Scale of cultivation**	**Urine concentration**	**Main observation**	**Reference**
indian carp and freshwater prawn	holding tanks, 120 days	0.01%	0.01% urine produced fish yield lower than cow manure or cow manure mixed with human urine	Jana *et al.* (2012a)
microalgae zooplankton tomato	hydroponic cultivation, 114 days	2%, 2%+Fe	culturing algae, zooplankton and tomatoes in an aquaculture system using only diluted human urine with iron amendment is possible	Adamsson (2000)
phytoplankton	5000 L tanks, 16 weeks	0.02%	primary production of phytoplankton was the highest with highest phosphate concentration (stored urine)	Jana *et al.* (2012b)
zooplankton	4500 L tanks, 10 days	0.01%	the best zooplankton growth was obtained in diluted human urine	Golder *et al.* (2007)
water spinach	hydroponic cultivation, 21 days	10%, 5%, 3.3%, 2%	2% urine produced comparable growth characteristics to those in nutrient solution	Yang *et al.* (2015)

Note: [a]reported as gN/m^2; [b]180-260 mgN per 0.4 kg pot; [c]reported as L/ha; [d]1530 mgN per 9 kg pot

2.1.4 Urine in Cultivation of Microalgae

Microalgal cultivation for biofuel and bioenergy feedstock purposes has become increasingly relevant as new energy sources are explored for. After harvesting, microalgal biomass can be utilized e.g. in anaerobic digestion to produce methane (for a review, see e.g. Sialve *et al.* 2009), in biodiesel production by extracting the algal lipids (Griffiths and Harrison 2009, for a review, see Chisti 2007), or as another type of products, such as nutritional supplements, natural pigments, or aquaculture feed (Vandamme *et al.* 2013). When using urine for algae cultivation, factors such as light intensity, possible water source, cultivation strategies (batch vs. continuous, open pond vs. closed photobioreactor, etc.), climate conditions, existing infrastructure and logistic considerations, additional supply of carbon dioxide (CO_2), algal harvesting, and biomass post-processing affect the cultivation system (e.g. sustainability) (Chisti 2007, Cho *et al.* 2013, Rawat *et al.* 2013, for a review, see e.g. Lam and Lee 2012a). One of the main issues affecting the economics of algal cultivation, i.e. the capital and operating costs as well as well as the viability of commercial production (Jegathese and Farid 2014), is the source of phosphorus and nitrogen; micronutrients are also required at lower concentrations. Sustainability issues impact on the cultivation of algal biomass for biofuel production: on one hand, nitrogen fertilizer manufacturing process is energy-demanding and causes greenhouse gas emissions, on the other hand the world's phosphorus reserves are depleting due to phosphate rock mining (Dawson and Hilton 2011, Hilton and Dawson 2012, Canter *et al.* 2015).

Microalgal Nutrient Sources

Different types of recyclable sources of nutrients in microalgal cultivation have been suggested (**Table 2.6**), and studied, mainly in laboratory scale. Microalgal cultivation in wastewater can be used to remove nutrients from the wastewaters and simultaneously grow biomass for further uses (e.g. Li *et al.* 2011, Lam and Lee 2012a). Suggestions to integrate algal cultivation to wastewater treatment with CO_2 supplementation have been made (Cho *et al.* 2013, Soares *et al.* 2013) as microalgae can fix CO_2 from industrial exhaust gases in the form of soluble carbonates (Sydney *et al.* 2010). On the other hand, life cycle analyses have demonstrated (e.g. Clarens *et al.* 2010, Medeiros *et al.* 2015) that cultivation of microalgae in wastewater still has its pressure points (the use of electricity, fertilizers etc.) that need to be addressed. Wastewater has been considered as an attractive and economic alternative for microalgal fertilizer source, but it is susceptible to virus and bacterial contamination, and in the worst-case scenario the whole microalgal colony could be devastated by other microorganisms thus leading to annihilation of microalgal population (Lam and Lee 2012a).

The use of human urine in microalgal cultivation has only been studied quite recently (**Table 2.6**). Human urine contains sufficient nutrients (especially N and P, **Table 2.2**) to support algal growth in addition to being a renewable resource. It has been hypothesized that urine used in cultivations should be nitrified (Feng *et al.* 2008) or stored (Jana *et al.* 2012b) to lessen the effect of ammonium inhibition or to inactivate microorganisms. Stored urine has less *Escherichia coli* than fresh urine (Jana *et al.* 2012b), whereas nitrification requires constant

pH-adjustment which increases expenses (Feng *et al.* 2008). During nitrification, ammonia volatilization can occur, affecting negatively on the ammonium-N concentration, which is why dilution of nitrified urine would be needed (Feng *et al.* 2007, Jana *et al.* 2012a). However, as urine quickly undergoes hydrolysis, leading to ammonium formation and precipitation of key nutrients (Zhang *et al.* 2014), the use of fresh urine is the most promising nutrient source in algal cultivation. Source-separated, hydrolyzed urine (urine was diluted to 3.5%) has been compared to other bioenergy feedstocks for algal cultivation (Clarens *et al.* 2010), but hydrolyzed urine needs excessive dilution, making it more water intensive than coupling algal cultivation with wastewater treatment. In addition, some microalgal strains are not able to re-grow efficiently in recycled water due to the susceptible contamination by fungus and bacteria (Lam and Lee 2012b).

TABLE 2.6. Different recyclable nutrient sources for microalgal cultivation, algal strains used, and the experimental scale.

Microalgal nutrient source	Algal strain	Cultivation volume and type	Reference
wastewater	-	-	Pittman *et al.* (2011)
agricultural	*Botryococcus braunii*	500 mL, bubble-column, piggery	An *et al.* (2003)
	Chlorella vulgaris	250 mL, batch, piggery	Abou-Shanab *et al.* (2013)
	Chlamydomonas mexicana		
	Nitzschia cf. pusilla		
	Scenedesmus obliquus		
	Ourococcus multisporus		
industrial	*Chlorella vulgaris*	40 L, high rate algae pond, textile	Lim *et al.* (2010)
	Chlorella vulgaris	500 mL, photobioreactor, dairy waste	Abreu *et al.* (2012)
	Chlorella vulgaris	250 mL batch, oil industry waste	Wang *et al.* (2013)
municipal	*Chlorella* sp.	250 mL, batch	Li *et al.* (2011)
	Chlorella sp.	25 L, coil reactor	
	Chlorella vulgaris	500 mL, batch	Choi and Lee (2012)
	Chlorella vulgaris	1000 mL, semi-continuous	de-Bashan *et al.* (2004)
	Chlorella sorokiniana		
	Chlorella vulgaris	2 L, tubular photobioreactor	He *et al.* (2013a)
anaerobic digestion effluent	*Chlorella* sp.	250 mL, batch, dairy manure	Wang *et al.* (2010)
	Nannochloropsis salina	2 L, batch, semi-continuous	Cai *et al.* (2013)
	-	-	Sialve *et al.* (2009)
	Muriellopsis sp.	300 mL, bubble column	Morales-Amaral *et al.* (2015)
	Pseudokirchinella subcapitata		
food waste hydrolysate	*Schizochytrium mangrovei*	2 L, batch	Pleissner *et al.* (2013)
	Chlorella pyreidinosa		
	Chlorella vulgaris	150 mL, batch, darkness	Lau *et al.* (2014)
hydrothermal liquefaction aqueous by-product	*Chlorella vulgaris*	500 mL, batch	Barreiro *et al.* (2015)
	Nannochloropsis gaditana		
	Phaeodactylum tricornutum		
	Scenesdesmus almeriensis		
	Desmodesmus sp.	2 L, batch	Alba *et al.* (2013)

Note: - review study, not reported

TABLE 2.6. Continues.

Microalgal nutrient source	Algal strain	Cultivation scale	Reference
struvite	*Chlorella vulgaris*	10 L, batch, photobioreactor	Moed *et al.* (2015)
human urine	*Spirulina platensis*	1.2 L, batch, bubble column	Feng *et al.* (2007)
		1 L, batch, photobioreactor	Yang *et al.* (2008)
		1.2 L, bubble column	Feng *et al.* (2008)
		1.2 L, batch, bubble column	Chang *et al.* (2013)
		1.2 L, photobioreactor	Feng and Wu (2006)
	Chlorella sorokiniana	microtiter plate	Tuantet *et al.* (2014a)
		1 L, photobioreactor	Tuantet *et al.* (2014b)
		6 L, photobioreactor	Zhang *et al.* (2014)
	Scenedesmus acuminatus	40 L, greenhouse culture	Adamsson (2000)

2.2 Pharmaceuticals and Estrogenic Hormones in Urine

To make utilization of urine possible, besides factors such as the varying collection, storage, and transportation methods, also the possible occurrence of harmful substances in urine and their fate must be considered. Pharmaceutical residues and hormones are excreted through human urine and therefore, use of urine is associated with potential transfer of pharmaceutical residues to the environment, agricultural fields, crop plants and thus, humans (Winker 2010). The effect of estrogenic compounds on endocrine and reproductive systems of wildlife (e.g. fish feminization, Larsson *et al.* 1999) has been noted (Soto *et al.* 1995, Routledge *et al.* 1998), and female sex-hormones and synthetic steroids are the most potent estrogenic compounds regarding their endocrine-disrupting properties (Rodriguez-Mozaz *et al.* 2004b). In addition, many compounds that are found in the environment have estrogenic properties: certain pesticides and herbicides, some polychlorinated biphenyls (PCBs), plasticizers, breakdown products of surfactants, and phthalates (Routledge and Sumpter 1996, Beck *et al.* 2006). In a wastewater treatment system, conjugated pharmaceuticals and hormones can transform back into their (biologically) active forms by microbial metabolism already in the sewer network or at the WWTP (Desbrow *et al.* 1998, Jelíc *et al.* 2015) and be transported to watersheds.

Pharmaceutically active ingredients comprise a large variety of chemical compounds (more than 3 000 registered in the EU alone) which have different therapeutic modes of action, different physico-chemical properties and susceptibilities to (biological) degradation (Kujawa-Roeleveld *et al.* 2008, Kümmerer 2009). Thus, one important factor in pharmaceutical and estrogenic monitoring is the use of applicable detection methods. Next, more insight is given on specific compounds and estrogenic hormones (**Chapter 2.2.1**), their fate in urine and wastewater treatment processes (**Chapter 2.2.2**), and the analytical methods for detecting and monitoring them in urine (**Chapter 2.3**).

2.2.1 Pharmaceuticals, Estrogens, and Their Excretion in Urine

Pharmaceuticals and hormones, as well as nutrients (**Table 2.2**) are excreted to a large extent into urine (Winker *et al.* 2008a, 2008b, **Table 2.7**), the production of which is 1.2 L/person/day on average. Nutrients, hormones, and microorganisms (bacteria, viruses) are naturally present in collected urine, as described in **Chapter 2.1**, while pharmaceuticals and the transformation products have been present in urine largely since the 1970's, when the concept of medicalization entered the modern society (Becker and Nachtigall 1992). Many pharmaceutical compounds go through a structural change in the human body, due to organisms in the gut or by human enzymes (Pelkonen and Ruskoaho 2003). This structural change turns the compounds into metabolites, which do not make a difference between biochemical processes, bacterial activity, or hydrolysis (Längin *et al.* 2008, Kümmerer 2009).

At the beginning of the 21st century, several groups of pharmaceuticals considered environmentally relevant were listed (Kümmerer 2001, Jain *et al.* 2013 and references therein): (*i*) antiviral compounds, due to their transportation in the food chain, their escapement of degradation at WWTPs, and interference with the natural biological systems of living organisms, (*ii*) antibiotics, because of their pronounced bacterial toxicity and their potential of fostering resistance, and (*iii*) hormones, because of their high efficiency/low effect threshold. Compounds from all of these pharmaceutical groups can be detected in human urine.

The antibiotic (*ii*) resistance reported at WWTPs is widely recognized (Xu *et al.* 2015), whereas antiviral drugs *(i)* have lately been gaining attention as possible environmental pollutants and possibly inducing antiviral drug resistance among influenza viruses (Prasse *et al.* 2010, Jain *et al.* 2013). Antibiotics can also negatively affect plant growth and soil microbial and enzymatic activities (Liu *et al.* 2009). Studies have shown that irrigation with pharmaceutical-containing reclaimed water leads to presence and accumulation of pharmaceuticals in soil (Kinney *et al.* 2006) and urine fertilization may lead to e.g. carbamazepine transportation into the roots and aerial plant parts of ryegrass (Winker *et al.* 2010). Steroidal estrogenic hormones (*iii*) have been recognized as environmental pollutants already a few decades ago (Shore *et al.* 1993) and they have been detected in surface and wastewaters (Vermeirssen *et al.* 2005) while evidence of their presence in the environment is continuous (e.g. soils, Goeppert *et al.* 2015). The following list presents some selected compounds from the three previously described categories *i-iii* (studied in this thesis), considered of environmental concern due to their poor degradability, bacterial or viral resistance, and low threshold effect in the environment (see **Tables 2.7** and **4.3** for more information):

- *(i)* nevirapine (NVP), lamivudine (3TC), and zidovudine (ZDV), that are commonly prescribed as antivirals to treat HIV infections (therapeutic group of antiviral compounds),

- *(ii)* rifampicin (RMP) and ciprofloxacin (CIP) that are widely used to treat tuberculosis (therapeutic group of anti-tuberculotics),

- *(ii)* trimethoprim (TRI) and sulfamethoxazole (SMX) that are used in combination with HIV drugs to prevent infectious diseases in HIV patients (therapeutic group of antibiotics),

- carbamazepine (CBZ), one of the most important antiepileptic drugs (Bertilsson and Tomson 1986), and

- *(iii)* estogens, namely estriol (E_1), 17β-estradiol (E_2), synthetic 17α-ethinylestradiol (EE_2) and estrone (E_3).

TABLE 2.7. Selected pharmaceuticals (studied in this thesis), their uses and excretion as a parent compound and metabolites in urine.

Pharmaceutical	**Use**	**Excretion in urine as a parent compound** (%)	**Reference**
Lamivudine (3TC)	treatment of HIV, antiretrovirals [a]	70	Atkinson *et al.* (2007)
Zidovudine (ZDV)		14–20	Johnson *et al.* (1999)
Nevirapine (NVP)		2.7	Smith *et al.* (2001)
Sulfamethoxazole (SMX)	sulphonamide antibacterials, used in treating urinary tract infections, pneumonia, bronchitis, meningitis, or toxoplasmosis, and in veterinary medicine. Also called co-trimoxazole[b], TRI and SMX are most likely used to treat HIV-related pneumonia.	14–48	Fernandez-Torres *et al.* (2010)
Trimethoprim (TRI)		37–81	Johnson *et al.* (1999)
			Pérez *et al.* (2005)
			Patel and Welling (1980)
Ciprofloxacin (CIP)	fluoroquinole antibacterial used e.g. in tuberculosis treatment (commonly associated with HIV infection). Also an effective antibiotic against *Proteus mirabilis*, a common bacterium in the urinary tract that can cause urinary infections and formation of urinary crystals.	40–50	Vance-Bryan *et al.* (1990)
Rifampicin (RMP)	rifamycin antibacterial. Used in the treatment of tuberculosis, caused by *Mycobacterium tuberculosis.*	13–24	Burman *et al.* (2001)
Carbamazepine (CBZ) [c]	antiepileptic. CBZ is a persistent pharmaceutical and is not subjected to any degradation or adsorption neither in wastewater treatment nor in soils.	3	Clara *et al.* (2004)
			Gibson *et al.* (2010)
Estrogens	**Use**	**Elimination as glucuronides** (%)	**Reference**
Estrone (E_1)	estrone is produced primarily in the ovaries, placenta, and in peripheral tissues (especially adipose tissue)	85–89	DrugBank (2015)
			Roche Diagnostics GmbH (2014)
17β-Estradiol (E_2)	estradiol is the most potent form of estrogenic steroids. In humans, it is produced primarily by the ovaries and the placenta. It is also produced by the adipose tissue of men and postmenopausal women.	90–95	
17α-Ethinylestradiol (EE_2)	semisynthetic and has high estrogenic potency when administered orally. EE_2 is often used as the estrogenic component in oral contraceptives.	90–95	
Estriol (E_3)	estriol is a major urinary estrogen, large amounts of which is produced by the placenta during pregnancy.	90–95	

Note: [a] 3TC, ZDV and NVP are used in combination in HIV treatment as a fixed-dose combination tablets (Marier *et al.* 2007). [b] Co-trimoxazole is also effective against *P. mirabilis*. [c] CBZ has been used as a marker for a non-biodegradable substance and it has been detected in many WWTP effluents.

After administration or production and/or synthetization in the human body, pharmaceuticals and estrogens are metabolized and excreted mostly in urine via kidneys (or through bile and via faeces), partially as parent compounds and as a number of inactive and active metabolites with modified chemical structures (Pelkonen and Ruskoaho 2003, Kujawa-Roeleveld *et al.* 2008, Schürmann *et al.* 2012). The excretion varies for the previously presented compounds (**Table 2.7**) in the range of 2.7–70% as parent compounds; for estrogens, excretion is based mainly on glucuronides, which comprise approximately 85–95% of the excreted concentration (**Table 2.7**). As estrogens can be less bioactive in their glucuronide-form, to enhance analysis efficiency, the chemical glucuronides must be broken down to release the active, original compound (**Figure 2.1**).

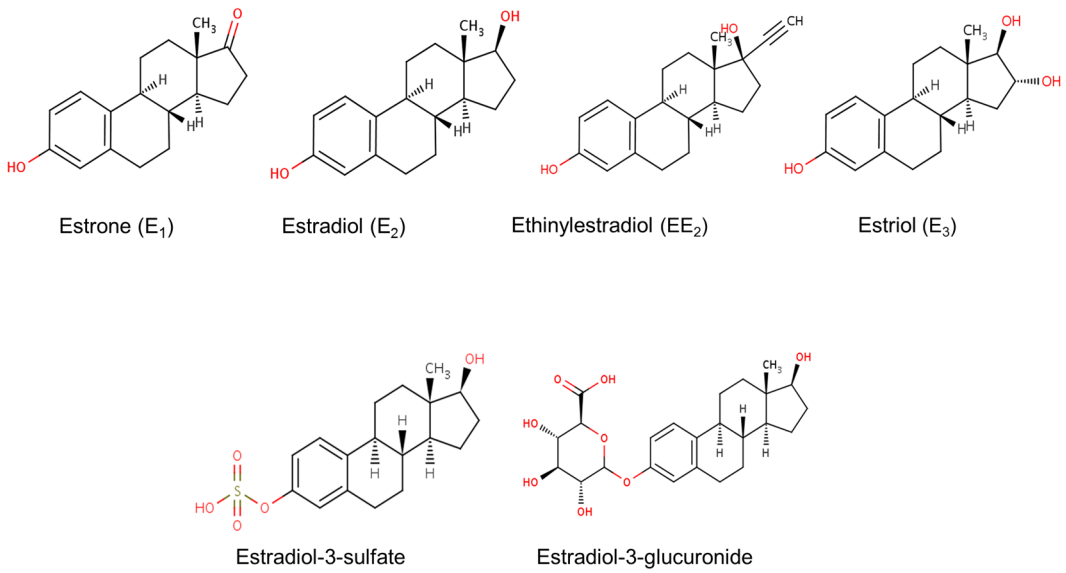

FIGURE 2.1. Chemical structures of the four estrogens (DrugBank 2015) and the conjugated forms of estradiol as examples of urinary estrogen conjugates (ChEBI 2013, MolDB 2013).

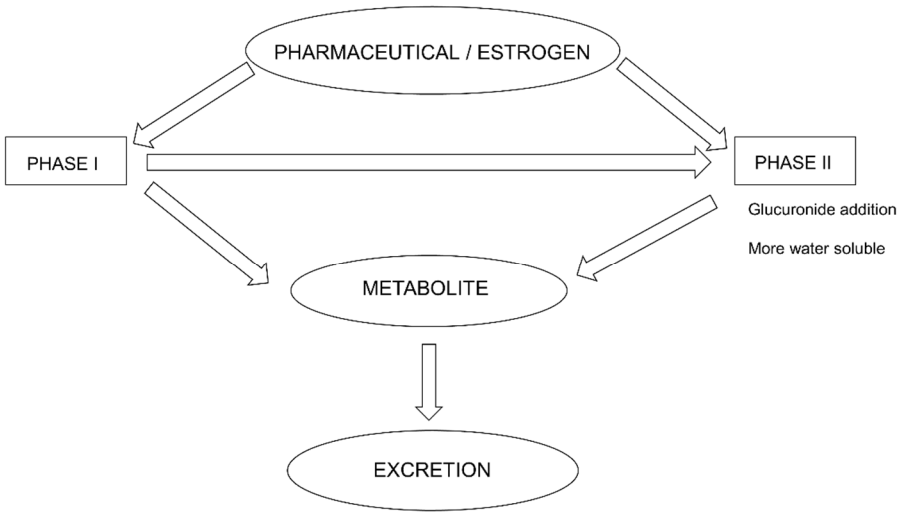

FIGURE 2.2. An overview of the metabolism of a parent compound into phase I and phase II metabolites (adapted from Halling-Sørensen *et al.* 1998, Koulu and Tuomisto 2007).

In human metabolism (of pharmaceuticals and hormones) four different end-products are observed: an active compound results in inactive metabolite (*i)*, an active compound results in active metabolite (or both active and inactive metabolites) *(ii)*, an inactive compound results in active metabolite (*iii*), or an inactive compound results in toxic metabolite (*iv*) (Koulu and Tuomisto 2007). As a result of conjugation, the compound gains a glucuronide, glutathione, acetyl, or a sulfate group (Pelkonen and Ruskoaho 2003) and is then excreted into urine. Metabolism can be divided into two phases describing the water solubility of the metabolite (**Figure 2.2**). Both phase I and phase II reactions depicted in **Figure 2.2** change the physical behavior of the substance, since metabolism always renders the metabolites more water-soluble than the parent compound (Halling-Sørensen *et al.* 1998); phase II metabolites being most water-soluble. In the intestine or in urinary bladder de-conjugation reactions can occur, which leads to release of parent compound or phase I metabolite into urine (Pelkonen and Ruskoaho 2003). The presence of metabolites in urine is essential relating to urine-based fertilizers, as metabolites are susceptible to change back to original, active forms of the compounds e.g. in the WWTPs and in the soil (Kurisu *et al.* 2010, Goeppert *et al.* 2014, Jelíc *et al.* 2015), thus posing a risk to fertilization applications.

2.2.2 Fate of Pharmaceuticals and Estrogens in Urine Treatment and in Wastewater Treatment Plants

In order to understand the effect of urine removal from municipal wastewater, the presence of pharmaceuticals and estrogens at WWTPs and receiving waters needs to be assessed. Pharmaceuticals and their metabolites are excreted mainly via urine (only partly via feces), as discussed earlier (**Chapter 2.2.1**) and thus the potential fate of pharmaceuticals and their metabolites is defined by the following processes. For example, in the flush sewage systems urine is led into municipal WWTPs, while in other systems urine may be discharged directly into environment (Escher *et al.* 2005, 2006). One major difference between source-separating urine and treating wastewaters at WWTPs is that in the former the urine has a significantly higher concentration of pharmaceuticals (µg/L or mg/L vs. ng/L measured at WWTPs) as well as bulk organics and nutrients (Kujawa-Roeleveld *et al.* 2008).

Finding processes to remove pharmaceuticals and hormonal compounds from urine is of notable importance considering its re-use (Gajurel *et al.* 2007). Among the different techniques investigated for urine treatment and pharmaceutical removal (**Tables 2.4** and **2.8**), nanofiltration, struvite precipitation, and electrodialysis are all effective in separating inorganic nutrients from pharmaceuticals for further applications, while a supplemental treatment is recommended to remove or degrade the pharmaceuticals (Dodd *et al.* 2008). Ozonation is not considered as a process for fertilizer production, but it can be used as an additional urine treatment step to break down pharmaceutical compounds (Escher *et al.* 2006). It is also possible, in principle, to remove pharmaceuticals by adsorption to active carbon or other adsorbents (Maurer *et al.* 2006), but the high organics concentration in urine will probably interfere with the adsorption process (Quinlivan *et al.* 2005).

TABLE 2.8. Comparison of different urine treatment processes for pharmaceutical elimination.

Technique	Waste product	Fertilizer product	Estrogenicity removal	Removal of single compounds	Reference
electrodialysis	diluate, drugs	concentrate	99.7% 86–94%	EE_2 > 98% ibuprofen >95%	Escher *et al.* (2006) Pronk *et al.* (2007)
bioreactor (after 24h)	-	treated effluent (stabilized urine)	E_2 92% EE_2 73%	E_2 89% EE_2 55% propranolol 0% ibuprofen 0%	Escher *et al.* (2006)
nanofiltration	concentrate drugs	permeate	53.4%	propranolol 59% ibuprofen 96% diclofenac 96% carbamazepine 74% EE_2 96%	Escher *et al.* (2006)
		-	-	EE_2, diclofenac, ibuprofen, propranolol, carbamazepine: at pH 5.3, >92% removal; at pH 4.8, 73% removal	Pronk *et al.* (2006)
struvite precipitation	filtrate, drugs	struvite	>98.1%	EE_2 and E_2 < LOD propranolol 99.2% ibuprofen >99.9% diclofenac >99.9% carbamazepine 99.7%	Escher *et al.* (2006) [a]
		washed struvite	-	diclofenac <LOD hydrochlorothiazid <LOD metoprolol <LOD nebivolol <LOD	Schürmann *et al.* (2012)
		washed struvite	-	E_1 97% E_2 95% EE_2 100% propranolol 94.2% ibuprofen 99.6% diclofenac 99.9% carbamazepine 96.4%	Ronteltap *et al.* (2007)

Note: [a] struvite dissolved after washing; <LOD - concentration below limit of detection; - not reported

TABLE 2.8. Continues.

Technique	Waste product	Fertilizer product	Estrogenicity removal	Removal of single compounds	Reference
ozonation	-	-	>99% (1.1 gO_3/L)	removal <LOD (0.6-0.8 gO_3/L) for EE_2, propranolol, diclofenac, carbamazepine and at 1.3 gO_3/L for ibuprofen	Escher *et al.* (2006)
	-	-	-	ibuprofen 50%, clofibric acid 50%, carbamazepine 95%, diclofenac 95%	Gulyas *et al.* (2007)
	-	-	-	ibuprofen 15–117%, bezafibrate 17–97%, β-sitostrerol 32–79%, carbamazepine 67–92%, diclofenac -31–151%, pentoxifylline 69%	Tettenborn *et al.* (2007)[b]
	-	-	-	>99% EE_2 at 0.06 gO_3/L	Dodd *et al.* (2008)
steam stripping	N-depleted urine	ammonium-containing condensate	-	>99% for ibuprofen, bezafibrate, β-sitosterol, diclofenac, phenacetin, carbamazepine, pentoxifylline	Tettenborn *et al.* (2007)
evaporation	distillate	concentrate	-	in 3.5 times concentrated urine phenacetin, carbamazepine, pentoxifylline, phenazone were detected, 50% of bezafibrate, 30% of diclofenac and ibuprofen,	Tettenborn *et al.* (2007)
UV-radiation	-	-	-	ibuprofen 46–93%, bezafibrate 42–348%, β-sitosterol -58–38%, carbamazepine 74–113%, diclofenac 37–72%, pentoxifylline 48–87%	Tettenborn *et al.* (2007)[c]
	-	-	-	degradation of sulfamethoxazole and trimethoprim, formation of metabolites of different properties	Zhang *et al*. (2015)[d]

Note: UV – ultraviolet; O_3 - ozone; - not reported; [b] removal depending on gO_3/L supplied; [c] removal depending on efficiency of UV-radiation; [d] synthetic fresh and hydrolyzed human urine

While the fate of pharmaceuticals and estrogens in the treatment or use of source-separated urine are little investigated, their fate at WWTPs has been intensively studied and in the following some general overview of the compounds studied in this thesis is given (**Tables 2.9** and **2.10**). Over the years, several studies investigating the pharmaceutical concentration at current WWTPs have shown the inconvenient truth: most pharmaceuticals are only partly removed during treatment processes (Ternes *et al.* 2001, Ternes *et al.* 2004, Blair *et al.* 2015) and some appear quite persistent (Clara *et al.* 2004). In **Tables 2.9** and **2.10**, the average concentrations reported in the literature for the selected compounds are given. The fate of the studied compounds in the WWTP process has varied; e.g. 3TC is reported to be eliminated more than 90%, while removal of NVP and ZDV has been negligible (**Table 2.9**). According to a review by Jain *et al.* (2013), antiviral compound persistence in WWTPs can be observed with other antiviral compounds as well. It has been claimed that pharmaceuticals are transformed back to their active forms by the WWTP microorganisms (Jelíc *et al.* 2015), thus explaining the larger concentration leaving from the WWTP than what is recorded in the influent. In the case of estrogens, the removal percentages at WWTPs have varied from -62.4% to 98.8% in the reported investigations (**Table 2.10**). The natural estrogens E_2 and E_3 are eliminated by 44–96%, and E_1 is not removed in some cases at all, while the synthetic EE_2 used in e.g. contraceptives is 34–43% removed.

TABLE 2.9. Pharmaceutical concentrations in WWTP influents and effluents, and their removal percentages reported in the literature. In some cases, concentrations are from grab samples, in others 24-h collection samples. The removal is expressed as a difference between the influent and the effluent, while the fate is not presented. The values are mean concentrations.

	At WWTP (ng/L)			
Analyte	Influent	Effluent	Removal (%)	**Reference**
Lamivudine	210–720	< LOQ	76–93	Prasse *et al.* (2010)
Nevirapine	4.8–21.8	7.2–32.1	0	Prasse *et al.* (2010)
Zidovudine	310–380	98.2–564	0–68	Prasse *et al.* (2010)
Sulfamethoxazole	-	-	-138–60	Göbel *et al.* (2007)
	580	250	43	Carballa *et al.* (2004)
	144–674	135–304	6–55	Lindberg *et al.* (2005)
	800–2200	900	40	Pérez *et al.* (2005)
Trimethoprim	-	-	-40–20	Göbel *et al.* (2007)
	99–1300	66–1340	-3–33	Lindberg *et al.* (2005)
	300–500	100	75	Pérez *et al.* (2005)
Ciprofloxacin	200–600	29–40	86–93	Vieno *et al.* (2006)
	<LOD–4230	<LOD–130	97–100	Vieno *et al.* (2007)
	102–300	7–18	93–94	Lindberg *et al.* (2005)
Rifampicin	-	-	-	-
Carbamazepine	160–820	290–2440	-81– -198	Vieno *et al.* (2007)
	290–400	380–470	-18– -23	Vieno *et al.* (2006)

Note: A negative value in the elimination-column means that effluent had higher concentration than influent. In most cases, the variation is reported between different seasons or different WWTPs and sampling sites; - not reported

TABLE 2.10. Estrogen concentrations in surface waters and in WWTP influents and effluents reported in the literature. In some cases concentrations are from grab samples, in others 24-h collection samples. The values are mean concentrations.

	Estrone E_1	**17β-estradiol** E_2	**17α-ethinyl-estradiol** EE_2	**Estriol** E_3	**Reference**
surface water (ng/L)	-	2.2–2.6	0.48–0.52	-	Snyder *et al.* (1999)
	1.1–3.0	1.4–3.0	1.1–2.9	1.0–2.5	Cargouët *et al.* (2004)
	0.1–4.1	0.15–3.6	0.1–5.1	-	Kuch and Ballschmiter (2001)
	< 0.1–2.9	< 0.3–5.5	< 0.1–4.3	-	Belfroid *et al.* (1999)
WWTP influent (ng/L)	-	0.71	-	-	Snyder *et al.* (1999)
	19–78	2.4–26.0	-	-	Servos *et al.* (2005)
	56.2	15.7	< 0.3	26.2	Fan *et al.* (2011)
	54.8	22	< 5.0	-	Braga *et al.* (2005)
	9.6–17.6	11.1–17.4	4.9–7.1	11.4–15.2	Cargouët *et al.* (2004)
WWTP effluent (ng/L)	-	0.45-3.66	0.24–0.76	-	Snyder *et al.* (1999)
	1–96	0.2–14.7	-	-	Servos *et al.* (2005)
	12.0	1.0	< 0.3	0.2	Fan *et al.* (2011)
	8.1	0.95	4.5	-	Braga *et al.* (2005)
	4.3–7.2	4.5–8.6	2.7–4.5	5.0–7.3	Cargouët *et al.* (2004)
	0.35–18	0.15–5.2	0.1–8.9	-	Kuch and Ballschmiter (2001)
	2.4	4.4	-	-	Carballa *et al.* (2004)
removal at WWTPs (%)	85	96	-	-	Braga et *al.* (2005)
	-62.4–97.8	39.5–98.8	-	-	Servos *et al.* (2005)
	44–59	43–60	34–43	40–67	Cargouët *et al.* (2004)

Note: In most cases the variation is reported between different seasons or different WWTPs and sampling sites; - not reported

Pharmaceuticals and Hormones in Soil and Transportation into Agricultural Products

One of the main concerns in using urine on agricultural purposes on edible plants is the leaching of these compounds in the soil and transportation and accumulation of the pharmaceuticals and hormones into the plants. Sorption is seen as the major factor influencing pharmaceutical behavior in the soil (Winker *et al.* 2009) when using reclaimed wastewater sources while using urine, a much lower concentration is sorbed onto soil particles (Kujawa-Roeleved *et al.* 2008). The organic matter content in urine decreases the soil capacity to sorb pharmaceuticals (Kujawa-Roeleved *et al.* 2008) and urine is not only affecting the sorption rate but prolongs vertical migration rates as well as hinders microbial degradation (Lucas and Jones 2009). In soils, the bioavailability is determined by the sorption characteristic by clay minerals and soil organic matter as well as soil pH (Carvalho *et al.* 2014). Behavior of estrogens in soil is dependent upon the aqueous matrix type where it is delivered to the soil (Lucas and Jones 2009).

The prior exposure of the soil to pharmaceuticals (i.e. has the pharmaceutical already been introduced to the soil microorganisms) can influence the degradative ability of the soil microbial community and be inhibitory to degradation – additionally, the effect of soil oxygen content plays a major role (Xu *et al.* 2009, Carr *et al.* 2011). Biological degradation under aerobic conditions in the soil can be more rapid or similar in comparison with unexposed soils for estrogens and some pharmaceuticals than in prior exposed soils (Carr *et al.* 2011). Ibuprofen, for instance, does not necessarily degrade under lower bacterial concentrations found in soil,

but requires high bacterial activity found in activated sludge WWTPs, whereas CIP does not degrade at all but is immobilized in the soil (Carr *et al.* 2011). Under anaerobic conditions, influencing factors such as nutrient availability, microbial population, and soil structure could have an effect (Carr *et al.* 2011). In the soil, microbial transformations, sorption, the capillary transport of water and pharmaceuticals, and the levels of pharmaceuticals in contact with roots may vary both spatially and temporally (Wu *et al.* 2013). Winker *et al.* (2010) have demonstrated that ryegrass can uptake CBZ on roots and aerial parts of the plant via urine fertilization, and the transport was driven by transpiration, while a cucumber can take up CBZ which can bioaccumulate at indigenous levels present in wastewater (Shenker *et al.* 2011).

The main factors affecting pharmaceutical and hormonal uptake in plants from soil are the acid dissociation constant (pK_a) and polarity of the compound (i.e. ionization at environmental conditions) – pharmaceutical translocation in the plant after root uptake is affected by water movement due transpiration, and hydrophilic compounds may have a greater movement potential (Herklozt *et al.* 2010, Wu *et al.* 2013). The plant uptake of pharmaceuticals during irrigation with treated wastewater may affect plant development indirectly by disrupting soil communities and soil function by decreasing the number of soil bacteria thus leading to lack of food for soil fauna (protozoa, nematodes, micro arthropods) (Fatta-Kassinos *et al.* 2011). Less data is available on pharmaceutical accumulation through urine fertilization but some studies have been conducted using e.g. reclaimed wastewater (Shenker *et al.* 2011) and nutrient solutions (Herklotz *et al.* 2010, Wu *et al.* 2013). For more information, see **Table 2.11** where some of the accumulation and leaching studies of pharmaceuticals and estrogenic hormones in the soil have been grouped.

The root uptake of pharmaceuticals has been shown to correlate positively with pH-adjusted octanol–water partition factor ($logK_{ow}$) for nonionic compounds whereas translocation from roots is negatively related (Wu *et al.* 2013). Ionization of compounds, including pharmaceuticals in the soil, is therefore important – weak acids or bases, for instance, undergo partial dissociation under environmental pH conditions and are thus present in soils in two or more forms, i.e. the neutral molecule and ionized species (Wu *et al.* 2013). Under soil pH conditions below their pK_a, organic acids are normally in their neutral and undissociated form, which has a higher tendency to sorb to soil organic matter (Xu *et al.* 2009). For ionizable organic compounds, ionization reduces their uptake to the shoots due to the decrease in the plant cell membrane permeability (Carvalho *et al.* 2014).

TABLE 2.11. Studies on pharmaceutical accumulation into plants and leaching of estrogens in the soil as reported in the literature

Source of pharmaceuticals and hormones	Pharmaceutical/ plant	Soil type (organic matter content %)	Type of experiment	Main observation	Reference
reclaimed wastewater	CBZ/cucumber	peat mixture (32.6) sandy soil (1.2) clay soil (6.5)	greenhouse, CBZ concentration 25 µg/L (spiked); 1 µg/L (reclaimed incl. 3 µg/L indigenous), 5 L pot, 50-60% water content	CBZ concentration in the fruit ranged 6.4–25.6 µg/kg.	Shenker *et al.* (2011)
nutrient solution	TRI, CBZ, SMX and 13 others/lettuce, cucumber, spinach, chili pepper	-	hydroponic cultivation, 21 days, 0.5 and 5 µg/L	vegetables were taking up pharmaceuticals. Among the studied pharmaceuticals, TRI (92–270 ng/g) accumulated in roots, CBZ in roots (2.9–67 ng/g) and leaves and stems (23–520 ng/g).	Wu *et al.* (2013)
nutrient solution	SMX, CBZ, TRI/cabbage	Hoagland's nutrient solution	hydroponic cultivation, 232.5 µg/L (spiked)	accumulation of pharmaceuticals in the roots (per wet weight): 98.87 ng/g CBZ, 138.26 ng/g SMX and 91.33 ng/g TRI.	Herklozt *et al.* (2010)
urine	CBZ, Ibuprofen /ryegrass	air-dried type Meckenheimer Krume (1.2)	greenhouse, 29 µg CBZ and 422 µg Ibuprofen (spiked) per pot, 9 kg pot, 80% water content	CBZ in aerial parts 4200–6950 µg/kg dry matter, roots 202–426 µg/kg dry matter. Ibuprofen was not transferred to the roots and probably degraded by soil bacteria.	Winker *et al.* (2010)
fertilized water	CIP, metformin, narasin/carrot, barley, wheat and 5 others	sandy soil (0.73)	greenhouse, 4 kg per pot, 10 mg/kg (spiked), duration 2–3 months, concentration in soil 6.5 mg/kg for CIP	in barley, CIP accumulated the least, while metformin was detected up to 0.1 mg/kg in the leafs and 1.5 mg/kg in the seeds.	Eggen *et al.* (2011)
spiked water	TRI and 9 others/carrot, lettuce	loamy sand soil (0.4)	greenhouse, 1 µg/kg, 1.5 kg soil per pot, 20% water content	TRI was detected in lettuce (6 µg/kg), carrot (5.3 µg/kg) and carrot peel (1.0 µg/kg).	Boxall *et al.* (2006)
sheep urine	estrone, estradiol	sandy clay loam textured Eutric Cambisol (2.1); sand textured Eutric Cambisol (2.7); sandy loam textured Haplic Podzol (1.2)	laboratory soil leaching study, 50 µg/L (spiked)	both estrogen and estradiol mineralization were significantly reduced in all soils. Addition of urine caused significant changes in microbial activity and their capacity to degrade estrogens.	Lucas and Jones (2009)

Pharmaceuticals and Hormones in Microalgal Cultivation

It has been shown that microalgal species (e.g. *Dunaliella tertiolecta*, *Selenastum capricornutum, Microcystis aeruginosa, Pseudokirchneriella subcapitata,* and some *Chlorella* sp.*)* are sensitive to antibacterial agents (Halling-Sørensen 2000, Yang *et al.* 2008, Qian *et al.* 2012) and pharmaceuticals and personal care products (DeLorenzo and Fleming 2008). Matamoros *et al.* (2015) have demonstrated that microalgal (incl. *Chlorella* sp.) uptake of organic compounds (including some pharmaceuticals) was indeed happening in a wastewater treatment system based on high-rate algal ponds. The most abundant compounds in the algal biomass where those with high $\log K_{ow} > 5$ (hydrophobic), and the removal of organic compounds from wastewater varied from 32% to 99% (Matamoros *et al.* 2015). When cultivating *C. vulgaris* with the common estrogens (E_1, E_2, EE_2, E_3), it was discovered that up to 9% of the hormones were partitioned within the algae (Lai *et al.* 2002). It was also proposed that *C. vulgaris* can biotransform the steroid estrogens (e.g. E_2 to E_1) and that some bioconcentration can occur (up to factor of 27) (Lai *et al.* 2002). Other microalgal strains (e.g. *S. capricornutum* and *Chladymonas reinhardtii*) have also demonstrated E_2 and EE_2 removal efficiency from culture medium while biotransformation products were also observed (Hom-Diaz *et al.* 2015). The effect of pharmaceuticals and hormones on algal growth and on biomass utilization options (Hom-Diaz *et al.* 2015) should therefore be studied further.

2.3 Methods for Pharmaceutical and Estrogenic Characterization in Urine

As described in the previous chapter, pharmaceuticals and hormones have been a rising matter of environmental concern, and them entering through urine into WWTPs and receiving waters have gained increasing attention. Pharmaceuticals are typically analyzed from waters, wastewaters, and urine using techniques that combine liquid chromatography (LC) with mass spectrometry (MS) techniques (e.g. Buiarelli *et al.* 2005, Mistri *et al.* 2007, Nebot *et al.* 2007, Prasse *et al.* 2010), providing information about the concentration of the analytes (at quite low ng/L concentrations) and also aiding to recognize unknown compounds via compound spectrum and mass libraries (Lai *et al.* 1997). Typically, LC-MS techniques are preceded by sample clean-up and concentration in order to reach equipment detection limits (Hao *et al.* 2007, Moreno-Bondi *et al.* 2009).

High performance (pressure) liquid chromatography (HPLC) analysis alone can provide a simple method for quantification of known pharmaceutical compounds in urine, especially at analyte concentrations >0.05 mg/L which is considered as a common detection limit for HPLC equipment. In the context of urine utilization, the need and interest to develop methods for the pharmaceuticals used in HIV treatment (studied in this thesis) rose from the fact that even though they are increasingly used globally, their environmental fate is far less studied than e.g. compounds such as painkillers, β-blockers, and lipid modifiers, of which a lot of analysis data can be found in the literature (e.g. Vieno *et al.* 2006, 2007, Winker *et al.* 2008a, 2008b).

However, methods for detecting the pharmaceuticals presented in **Chapter 2.2.1** are very diverse, are not necessarily optimized for urine matrix, and include only few of the compounds at a time from a selection of matrices. Therefore, a method able to detect the selected compounds in a single run would provide savings in analysis time and consumables.

Although LC and MS are very sensitive techniques, they are not suitable for screening purposes and do not consider the biological effects of a single hormone or chemical mixtures (Michelini *et al.* 2008). Endocrine disrupting compounds (EDCs, e.g. estrogens, estrogen-like compounds, plastic softeners, etc.) have several different chemical structures and mechanisms of action making their detection complicated using conventional, mainly chromatographic methods (Michelini *et al.* 2008). As the environmental concentrations of EDCs are quite low, sample matrices are complex, and the diversity of target compounds is vast, robust bioassays have been gaining interest (Salste *et al.* 2007). The advantage of biosensors in monitoring estrogenic activity is their capability to estimate cumulative estrogenic effects of a variety of chemicals in an environmental sample (Campbell *et al.* 2006) that correspond the overall activity of all estrogen receptor (ER) -binding chemicals present (Rehman *et al.* 1999). To identify different estrogenic compounds in environmental samples, the results from biological assays must be combined with gas chromatography (GC) or LC coupled to MS analyses (Salste *et al.* 2007).

2.3.1 Pharmaceutical Analysis of Urine with Liquid Chromatography

As already described, pharmaceuticals can be analyzed in urine with LC-technology. HPLC (operating pressure < 400 bar) and ultra-high performance liquid chromatography (UHPLC, operating pressure up to 1000–1200 bar) (Wren and Tchelitcheff 2006, Gritti and Guiochon 2010b, McCalley 2011) are common tools in sample analysis of different environmental and biological matrices (e.g. Jin *et al.* 2010, Núñez *et al.* 2012). HPLC is one of the main techniques, for example, in pharmaceutical development, where it is used to determine purity to control the quality and consistency of the active substance and dosage forms (Wren and Tchelitcheff 2006).

Core-Shell Columns vs. Conventional Columns

The 400-bar pressure limit of the conventional HPLC system has been a bottleneck for faster chromatographic analysis (MacNair *et al.* 1997). As the column particle size decreases, the backpressure of the system increases, and pressure in columns packed with 1-µm-diameter particles at its optimum flow rate is 125 times higher than in columns packed with 5 µm particles (MacNair *et al.* 1997). Commonly used column particle sizes are 3–5 µm and with complex samples, separation times of 30 minutes or longer are not uncommon (Wren and Tchelitcheff 2006): therefore, reducing separation time without reducing the separation quality has led to development of smaller particles which can be used with older HPLC equipment.

The porosity of the particles affect negatively on column efficiency at high flow rates and under high pressure gradients (Gritti and Guiochon 2010a): thus, the use of superficially porous

particles (aka core-shell particles) has the advantage of decreasing the thickness of the porous layer of the porous column packing material, which decreases the length along which molecules have to diffuse (Gritti and Guiochon 2010b, Ruta *et al.* 2012). Core-shell particles decrease the effect of the eddy diffusion (Gritti and Guiochon 2012) as the particle size distribution is very narrow (Ruta *et al.* 2012) and the particles cannot diffuse axially in the solid inner core (Ruta *et al.* 2012). Smaller particles allow higher flow rates while maintaining near-maximum efficiency (Fekete *et al.* 2011).

The use of core-shell columns with older HPLC equipment is well justified, as long as the extra-column contributions and system dwell volume are minimized (McCalley 2011), no methanol-water mixtures are used due to their approximately two-fold higher viscosity compared with acetonitrile (ACN)-water mixtures, and long columns providing high resolution are not required (Ruta *et al.* 2012). ACN has low viscosity which enables high elution rates (Gritti *et al.* 2010). With conventional HPLC, the performance of core-shell particles is roughly equivalent to that of sub-2 µm particles on a typical UHPLC (McCalley 2011).

Sample Pre-treatment in Chromatography and Simultaneous Analysis of Several Pharmaceuticals in Urine

Sample preparation is a fundamental step in analytical procedures as it helps to achieve detection limits as well as to extract, concentrate, and clean up samples (Bakkali *et al.* 1999, Pavlović *et al.* 2007), or to release the analyte from a conjugate (chemical or enzymatic hydrolysis) (Bakkali *et al.* 1999) with no need to dilute the sample (Fernandez-Torres *et al.* 2010). The most popular technique for liquid samples is solid phase extraction (SPE), which is extensively used to purify and concentrate samples containing several analytes from complex matrices (Fontanals *et al.* 2007), e.g. environmental and biological samples (Fontanals *et al.* 2007) and in toxicological analyses (Logan *et al.* 1990, Ferrara *et al.* 1992, Solans *et al.* 1995).

SPE sorbents come with different properties, but chemically modified polystyrene-divinylbenzene sorbents have been studied a lot (Fontanals *et al.* 2007), although for pharmaceuticals with $\log K_{ow}$ of 1.5-4.0, any reversed phase material should work (Pavlović *et al.* 2007). Matrix effects are a problem when extracting pharmaceuticals from urine, as they can influence the recovery efficiency and the extracted quantity (Pavlović *et al.* 2007). Sample pretreatment with SPE is suitable for urine, as it removes the by-products of urine matrix, such as inorganic salts and compounds which have low hydrophobicity (Escher *et al.* 2005).

As pharmaceutical compounds have a large variety of chemical structures (Pavlović *et al.* 2007, Fernandez-Torres *et al.* 2010), the choice of sorbent is a key factor affecting the selectivity, affinity, and sample capacity (Pavlović *et al.* 2007). Simultaneous analysis of e.g. antibiotics (Baranowska *et al.* 2006, Fernandez-Torres *et al.* 2010), β-blockers (Maguregui *et al.* 1995), and anti-inflammatory drugs (Hirai *et al.* 1997, Sun *et al.* 2003) from human urine and serum have been performed, but in several cases compounds have had similar physico-chemical properties. In many analytical methods, a compromise in the selection of

experimental conditions is generally required (Pavlović *et al.* 2007, Fernandez-Torres *et al.* 2010).

2.3.2 Bioluminescence Measurements of Estrogenic Compounds in Urine

In this chapter, the basic principle of bioluminescence measurements of estrogens with biosensors is given. In addition, the applicability of genetically modified, bioluminescent yeast biosensor in estrogen measurements in urine is shortly described.

Biosensors and Bioluminescence in Estrogenic Monitoring

Different biosensors include enzyme-, non-enzymatic-, immunochemical-, cell-, and receptor-based biosensors as well as DNA biosensors (Rodriguez-Mozaz *et al.* 2006, Rogers 2006). Biosensors include genetically modified microorganisms which give an observable response to target analytes (Rogers 2006) and are used for environmental monitoring such as toxicity, EDCs, biocides, hormones, PCBs, surfactants, antibiotics, and metals (D'Souza 2001, Rodriguez-Mozaz *et al.* 2004a).

Bioluminescence is light produced in a living organism by a chemical reaction which involves an enzyme/substrate system (Roda *et al.* 2009). Bioluminescent cell-based assays are simple to perform and have been used in bioanalytical measurements where high sensitivity is needed (Roda *et al.* 2009). A bioluminescent protein, an enzyme (luciferase, also β-glucuronidase, green fluorescent protein and β-galactosidase, Köhler *et al.* 2000), catalyzes the oxidation of the substrate (luciferin): one of the most commonly used proteins is luciferase obtained from a firefly *Photinus pyralis* (Roda *et al.* 2009). However, the exact reaction mechanism is yet not fully understood (Roda *et al.* 2009). Luciferases are also produced by other microorganisms, such as bacteria (e.g. *Vibrio fischeri* used in toxicity monitoring; for reviews, see e.g. Girotti *et al.* 2008, Roda *et al.* 2009).

Biosensors based on ERs provide information of the estrogenic potency of the samples (Rodriguez-Mozaz *et al.* 2004b). The principle of detection is based on the ER capturing the estrogen after which the complex is bound with an estrogen-responsive element immobilized on the sensor (Rodriguez-Mozaz *et al.* 2004b), which in turn, in the presence of appropriate substrate, produces light. The amount of reporter protein is proportional to the biological activity of the sample and can be measured by luminescence measurements (Michelini *et al.* 2008), while the obtained signal represents the sum activity of all estrogen receptor binding substances in the sample (Rehmann *et al.* 1999). Requirements to biosensors include simplicity, rapidness, cost-effectiveness, and field-portability, and they appear to be well suited to complement standard analytical methods for a number of environmental monitoring applications (Rogers 2006, Rodriguez-Mozaz *et al.* 2007). Biosensor advantages over chromatographic techniques include the redundancy of sample pre-treatment or concentration and sensitivity to a specific analyte due to biological recognition system (Leskinen *et al.* 2005, Rodriguez-Mozaz *et al.* 2007). Advantages of bioluminescence over other spectroscopic detection methods include higher detectability due to lower non-specific background signal and

the absence of interference from excitation light; bioluminescence-exploiting measurements also provide high sensitivity when the analyte concentration is low or sample size is very small (Roda *et al.* 2009); the analysis is robust and the sensor organisms can be grown on media devoid of steroids (Bovee *et al.* 2004a) when detecting hormonal compounds.

The quantitative analysis of all components in a complex sample is not possible (Rodriguez-Mozaz *et al.* 2007). Chemical analysis of all estrogenic compounds would be costly, and unknown metabolites and other estrogenic compounds can still be present in the (environmental) samples (Murk *et al.* 2002); therefore, knowing the sum of estrogenic activity in the sample can be more informative. Due to the great variety of compounds having estrogen-like activity, the classical instrumental analysis may not be the most suitable tool to assess estrogenicity of complex mixtures (Bovee *et al.* 2004a), such as urine. Biosensors may also be useful in a quick screening of endocrine-disrupting chemicals at municipal WWTPs, which has been previously performed e.g. by Desbrow *et al.* (1998) using a selection of sample pre-treatment steps.

Genetically Modified Yeast Cells in Estrogenic Monitoring in Urine

Bioluminescence tests performed on yeast cells are useful in the first stage screening as they are easy and inexpensive to perform and are more resistant to environmental contaminants than e.g. mammalian cells, which is an advantage when working with complex environmental samples (Leskinen *et al.* 2005), such as urine. A number of yeast tests for estrogenic compounds have been published, and many of them use the β-galactosidase enzyme as a reporter (**Table 2.12**). In addition, they take several days to perform due to long incubation times and sample pre-treatment requirements. (Routledge and Sumpter 1996, Bovee *et al.* 2005, Vermeirssen *et al.* 2005). However, bioluminescent yeast biosensors have not been previously used to detect estrogenic activity from source-separated human urine. Previously, human urine has been analyzed with biosensors mainly to produce information on steroid abuse (Zierau *et al.* 2008).

In vitro yeast estrogen screen (YES) assays utilizing β-galactosidase as a reporter have been successfully implemented in assessing estrogenic activity in environmental samples such as waters and wastewaters (Routledge and Sumpter 1996, Leskinen *et al.* 2005, Beck *et al.* 2006). A YES-based test battery has also been applied on human urine to detect toxicity of certain pharmaceuticals and to monitor the efficiency of wastewater (Escher *et al.* 2005) and urine treatment processes (Escher *et al.* 2006). YES responds to all substances with receptor-mediated estrogen activity regardless of the chemical structure, but the assay has a long induction time and assay protocol usually includes lysis of cells (Leskinen *et al.* 2005). The yeast biosensor utilizing luciferase as reporter protein (developed by Leskinen *et al.* 2003) was shown to respond to natural hormones and xenoestrogens similarly to the already established YES.

TABLE 2.12. Biosensors used in detection of estrogenic activity from different matrices.

Biosensor	Reporter protein	Detection	Matrix	Reference
ER-CALUX[a]	β-glucuronidase from *Escherichia coli* and *Helix pomatia*	bioluminescence	human urine	Legler *et al.* (2002)
			surface waters	Murk *et al.* (2002)
			WWTP influent, effluent, sludge, river water	
E-SCREEN[b]	-	cell yield	E_2 in EtOH	Soto *et al.* (1995)
Saccharomyces cerevisiae	β-galactosidase	fluorescence microscopy	E_2 in water	Wozei *et al.* (2006)
	β-glucuronidase	bioluminescence	WWTP effluent	Salste *et al.* (2007)
	β-galactosidase, β-glucuronidase green fluorescent protein	optical density luminescence fluorescence	standard solutions	Bovee *et al.* (2004a)
yeast estrogen screen (YES)	β-galactosidase	absorbance/ optical density	river water	Vermeirssen *et al.* (2005)
			surfactants and degradation products in EtOH	Routledge and Sumpter (1996)
			WWTP influent, effluent, sludge, river water	Murk *et al.* (2002)

Note: [a] estrogen-receptor (ER) mediated chemical-activated luciferase gene expression, human cell line; [b] human breast cancer estrogen-sensitive MCF-7 cells; EtOH - ethanol

The yeast biosensor is an attractive option for ER mediated activity screening in chemicals as well as in complex environmental samples and urine (Bovee *et al.* 2005, 2009, Leskinen *et al.* 2005). Androgen receptors have also been studied similarly to ERs in modified yeast cells (e.g. Michelini *et al.* 2008, Bovee *et al.* 2009). Bioluminescent yeast biosensors can be used to calculate the relative estrogenic potencies of estrogenic compounds to gain knowledge on their environmental effects (Bovee *et al.* 2004b, Leskinen *et al.* 2005). *Saccharomyces cerevisiae* can also be used to monitor non-specific toxicity in environmental samples (Välimaa *et al.* 2008). Yeast cells do not normally contain an ER, thus minimizing the concerns rising from interference from other gene targets, presence of other steroids, peptide hormones, or growth factors (Routledge and Sumpter 1996).

3 Aims of the Present Work

The main general objective of this thesis was to develop the use of source-separated urine. The main objective was further concretized to specific objectives of making characterization of urine easier and faster, to gain more understanding regarding pharmaceutical and estrogenic behavior during urine storage, and to evaluate the suitability of human urine in algal cultivations as a low-cost, renewable fertilizer. The specific aims of the present work were:

- To develop and apply analytical methods that make detection and quantification of pharmaceuticals and hormones in urine faster by
    - developing a method to detect several pharmaceuticals from human urine in a single six-minute analysis instead of analyzing each compound separately (**Paper I**).
    - assessing the feasibility of a yeast-cell-based biosensor in fast estrogenic activity detection in urine without any pre-treatment of the sample (**Paper II**).
- To assess the effect of the generally recommended six-month storage of urine on selected pharmaceuticals (**Paper III**).
- To evaluate the effect of urease inhibitor addition and fecal contamination in urine on pharmaceutical behavior during six-month storage (**Paper III**).
- To evaluate the feasibility of urine as nutrient source in microalgal cultivation and biomass production (**Paper IV**) by
    - comparing microalgal growth in diluted urine with growth in artificial growth medium.
    - screening the effect of urine dilution for maximum biomass production.
- To gain knowledge on microalgae nutrient utilization from urine (**Paper IV**).

4 Materials and Methods

This chapter describes the methods and analyses used in this thesis. The objectives as well as the methods are summarized in **Table 4.1**.

TABLE 4.1. Summary of the objectives and methods used in this thesis.

Objective	Method	Paper
method for analyzing several pharmaceuticals simultaneously in urine	development of an HPLC method able to detect eight pharmaceuticals in a single run in six minutes	**I**
method for fast estrogenic activity detection in both fresh and stored human urine	feasibility assessment of a yeast-cell-based biosensor without sample pre-treatment in detecting estrogenic activity in urine	**II**
pharmaceutical behavior in urine during a six-month storage	urine storage experiments for six months with pharmaceutical spiking and with feces/urease inhibitor amendment	**III**
urine as a nutrient source in microalgal cultivation	microalgal cultivation in different urine dilutions and as a reference in an artificial growth medium	**IV**

4.1 Substrate and Inocula

4.1.1 Urine Collection, Experimental Controls, and Pharmaceuticals Used

The human urine used in the experiments was collected from volunteers (28–38 years) in the Department of Chemistry and Bioengineering (Tampere University of Technology, TUT) as well as from four individuals outside TUT (9–38 years). A summary of the collected urine and different experimental set-ups are presented in **Table 4.2**. All volunteers were healthy and were taking no medication.

TABLE 4.2. Urine collection for each experiment conducted in this thesis.

Source of human urine	Processing before experiments	Reference
two persons: a male and a female	mixed	**Paper I**
six persons: a female, a female taking oral contraceptives, two males, a boy child, a girl child	used individually + mixed stored (one or five months) + mixed	**Paper II**
eight persons: both male and female	mixed	**Paper III**
one person: a male	sampled twice: one sample for each set-up	**Paper IV**

Urine used in the experimental controls (**Papers II-IV**) was not autoclaved, although autoclaving is often used to prevent biological activity. Preliminary tests indicated that autoclaving changed urine composition by transforming urea to ammonium, thus making use of such control unfeasible. Filtration of controls through 0.2 µm was excluded as it removes beneficial bacteria, and in a large scale, the costs would be high (**Papers II**, **III**, **IV**).

A method was developed to analyze several pharmaceuticals in urine simultaneously (**Paper I**), and the effect of urine storage on spiked pharmaceuticals was evaluated (**Paper III**). In addition, a bioluminescent whole-cell biosensor was used to determine the estrogenic activity from spiked standards and real human urine samples (**Paper II**). A summary of the physical and chemical properties of the pharmaceuticals and estrogens used in the experiments (**Papers I**, **II**, and **III**) are described in **Table 4.3** (next page).

TABLE 4.3. Physical and chemical properties of the pharmaceuticals, chemicals, and estrogens used in this thesis (**Papers I**, **II**, **III**).

Paper	Compound		Molecular weight (g/mol) [a]	pK_a [a,b]	$logK_{ow}$ [a,b]	Water solubility (g/L) [a,b]	Typical usage
I, III	*Pharmaceuticals*						
	Lamivudine	3TC	224.26	4.3	-1.29	2.76	antiviral (HIV-treatment)
	Zidovudine	ZDV	267.24	9.96	0.05	16.3	antiviral (HIV-treatment)
	Nevirapine	NVP	266.89	2.8	1.75	0.105	antiviral (HIV-treatment)
	Sulfamethoxazole	SMX	253.28	1.6; 5.7	0.89	0.459	antibiotic
	Trimethoprim	TRI	290.32	7.12	0.91	0.400	antibiotic
	Ciprofloxacin	CIP	331.35	6.09; 8.74	0.28	30	antibiotic (tuberculosis)
	Rifampicin	RMP	822.94	1.7; 7.9	4.24	1.4	antibiotic (tuberculosis)
	Carbamazepine	CBZ	236.27	13.9	2.45	0.0177	antiepileptic
II	*Estrogens*						
	Estrone	E_1	270.37	-5.3; 10.33	3.13	0.030	natural hormone
	17β-Estradiol	E_2	272.38	-0.88; 10.33	4.01	0.0036	natural hormone
	17α-Ethinylestradiol	EE_2	296.40	-1.7; 10.33	3.67	0.0113	synthetic hormone, used in contraceptives
	Estriol	E_3	288.38	-3.2; 10.33	2.45	0.0273	natural hormone

Note: Data collected from [a] DrugBank (2015); [b] Toxnet (2015).

4.1.2 Microorganism Strains and Cultivation Conditions Used

The microorganism strains, inoculum cultivation, and growth conditions used in this thesis (**Papers II** and **IV)** are described in **Table 4.4**. The yeast biosensor (developed by Leskinen *et al.* 2003, 2005) has been used in detection of estrogenic compounds from different matrices such as wastewaters and moisturizing lotions, but it has not been used with urine before (**Paper II**). Preparation of the medium and agar were based on Välimaa *et al.* (2008) and are described in detail in **Paper II**. *C. vulgaris* stock and maintenance cultures were grown in modified Chu-10 medium (originally by Stein (1973), modified by Acreman, for more detail, see **Paper IV**).

TABLE 4.4. Description of the inoculum and cultivation conditions used for *Saccharomyces cerevisiae* and *Chlorella vulgaris* (**Papers II** and **IV**).

	Estrogenic activity assessment of urine	**Microalgal cultivation in urine**
organism used	genetically modified bioluminescent yeast, *Saccharomyces cerevisiae BMAEREluc/ERα*	green microalga *Chlorella vulgaris* (SAG 211-11b)
inoculum origin	stored in glycerol stock at -80°C.	originally obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany.
inoculum cultivation	AHL-agar consisting of yeast nitrogen base w/o amino acids, agar-agar, glucose, and essential amino acids adenine, L-histidine and L-leusine (aka AHL).	the alga used had been sustained in maintenance cultures, from which inoculum were transferred into experimental cultivations.
growth conditions	inoculum was grown 48 h at 30°C after which one colony was transferred into 5 mL of liquid medium (AHL-medium without agar-agar), where it was incubated overnight at 30°C, 300 rpm.	culture was maintained and experiments conducted in 250 mL shake flasks at 24±1°C at 135 rpm under continuous 29 µmol photons/m^2/s illumination (5x OSRAM L 18W/965 Biolux fluorescent lamps) without CO_2 addition.
notes:	both the agar and liquid medium were autoclaved before glucose and amino acid addition at 121 °C for 15 min.	alga was grown in modified Chu-10 medium.
Reference	**Paper II**	**Paper IV**

4.2 Experimental Set-ups

4.2.1 Urine Storage Experiments (III)

For storage experiments, collected urine was combined thrice in a 5-liter volumetric flask, mixed and divided into 150 mL portions into sterile 60 plastic (polypropylene) bottles with tight lids (**Figures 4.1A** and **4.1B**). After sample division in experimental jars, pharmaceuticals (**Table 4.3**) were spiked into the samples at concentrations which might have be present in urine of a medicated person (the pharmaceutical concentration range of 7–140 mg/L/d for

antivirals, 43–469 mg/L/d for antibiotics, 60–300 mg/L/d for antituberculotics, and 20 mg/L/d for CBZ, **Paper III**). In short, each pharmaceutical was tested in laboratory storage, in triplicate, individually, in therapeutic groups, in therapeutic groups with feces addition, and in therapeutic groups with urease inhibitor addition. Urine was also stored without pharmaceuticals but amended with either feces or urease inhibitor.

In the beginning of the experiment and afterwards monthly for a duration of six months, pH and conductivity of the samples were measured and a 3 mL portion of the sample was filtrated for SPE pre-treatment. After SPE, samples were transferred into HPLC vials and run on Agilent 1100 HPLC with earlier developed method (both the HPLC method and SPE pre-treatment are described in detail in **Chapter 4.3.1** and in **Paper I**).

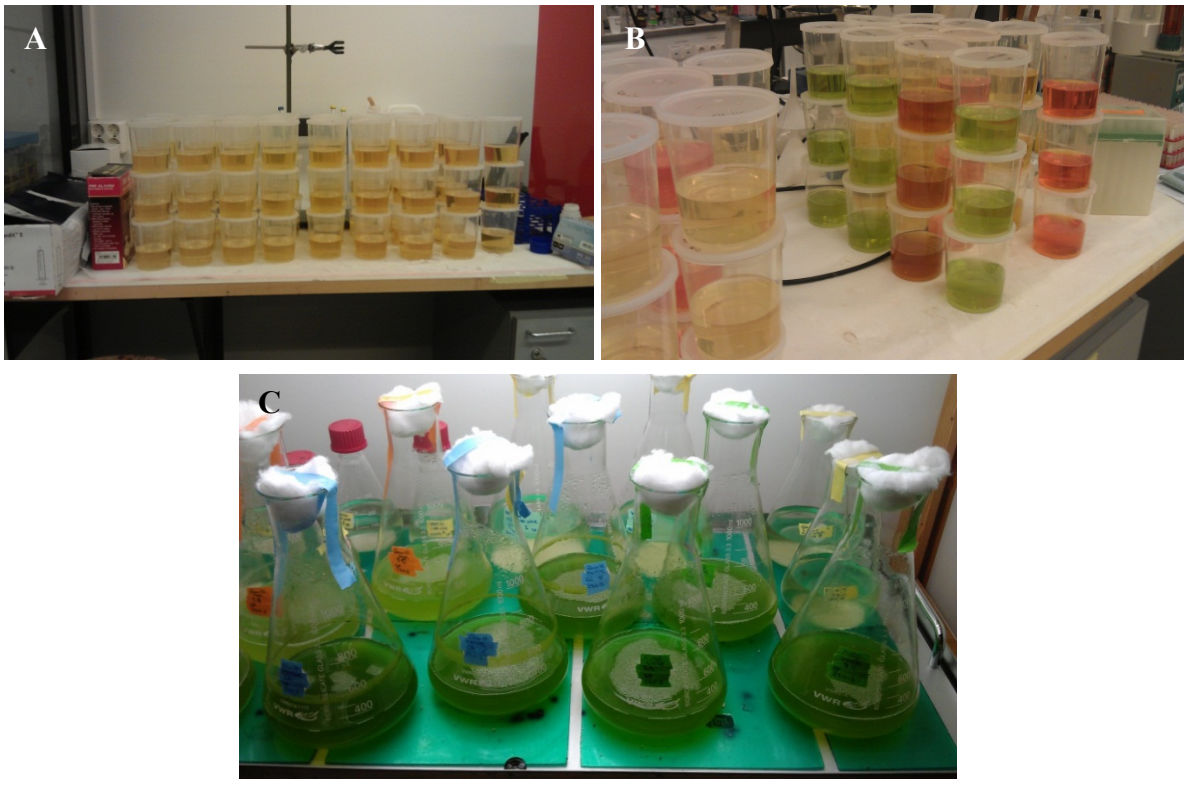

FIGURE 4.1. A) experimental set-up in storage experiments; mixed urine (150 mL) in 60 bottles, and B) same urine samples after pharmaceutical, feces, and urease inhibitor addition (green color is caused by urease inhibitor, orange by the antibiotic agent rifampicin) (**Paper III**). C) microalgal cultivation in batch bottles (**Paper IV**). The alga was cultivated in initial 600 mL of selected medium in 1 L Erlenmeyer shake flasks.

4.2.2 Microalgal Cultivation Experiments (IV)

Microalgal growth was studied in two experimental set-ups for 21 days each (**Paper IV**). One experiment was performed as triplicate in diluted urine (1:100), in modified Chu-10 medium, where the concentrations of N and P were adjusted to match to that of diluted urine, and in modified Chu-10 medium in which the N and P sources had been replaced by corresponding amount of urine but with all essential nutrients supplied as stock solutions. The other

experiment was conducted as duplicate, in different urine dilutions of 1:25, 1:75, 1:100, 1:150, and 1:300. Cultivation experiments took place in 1 L Erlenmeyer shake flasks (**Figure 4.1C**) in similar conditions as with stock and maintenance cultures (temperature, shaking, illumination, air supply).

4.3 Analytical Methods and Method Development

As eight different pharmaceuticals were analyzed, and many different chromatographic techniques were available for the selected individual compounds, there was a need to develop an analytical tool to detect all the compounds with a single method instead of having to analyze each compound separately. As for the estrogenic activity assessment, application of the yeast biosensor was hypothesized to provide rapid overview of the estrogenic activity in urine samples without having to perform time-consuming chromatographic analysis that would have required extensive sample pre-treatment steps.

4.3.1 HPLC Method for Simultaneous Detection of Pharmaceuticals in Urine (I)

A pre-treatment step for urine samples was needed in order to remove interfering substances before HPLC analysis. Thus, SPE with vacuum manifold was chosen as a cleaning step (for details, see **Paper I**). The SPE sorbent (Strata-X, 30 mg/33 µm) was conditioned with methanol (MeOH), equilibrated with MilliQ-water, and the filtrated (0.2 µm nylon) sample was loaded. After drying the sample for 1 minute in vacuum, sorbent was washed with 2% ammonium hydroxide to remove interferences, and to collect the fraction containing lamivudine (due to low logK_{ow}, **Table 4.3**), sorbent was dried for 1 min in vacuum and the rest of the sample was eluted with ACN:MeOH:Acetic acid (50:50:2, v/v/v). Method was validated for human urine by performing recovery tests for each pharmaceutical (**Paper I**).

An HPLC method was developed to monitor the concentrations of selected pharmaceutical compounds in urine during storage. In short, Agilent 1100 HPLC equipped with Kinetex XB-C_{18} 2.6 µm core-shell column (temperature 40°C) and a 0.2 µm pre-filter C_{18}-cartridge was run in a gradient mode: mobile phase consisted of ACN (eluent A) and 10 mM potassium phosphate buffer, pH 2.50 (eluent B). Gradient elution took place in 4.0 minutes: an isocratic hold from 0 min to 0.5 min at 5% eluent A, linear gradient from 0.5 min to 3.5 min from 5% to 70% eluent A, and an isocratic hold from 3.5 min to 4.0 min at 70% eluent A. The column was left to stabilize for 2.0 min after each sample, leading to a total analysis time of 6.0 min per sample. The detection wavelengths were 210 nm and 264 nm (diode array detector) and the flow rate used was 2.5 mL/min. The method was validated by performing repeatability and robustness tests (**Paper I**).

4.3.2 Estrogenic Activity Detection in Urine (II)

For the hormonal activity assays, yeast cells were grown to optical density (OD_{600}) of 3.0 and afterwards the culture was diluted with AHL-medium to OD_{600} of 0.8 and used in the assay with $_D$-luciferin solution (**Paper II**, developed by Leskinen *et al.* (2003)). The $_D$-luciferin solution was sterilized by sterile filtration and stored in 4 mL fractions in falcon tubes at -20°C covered with aluminum foil due to its light sensitivity. For analysis, one tube was unfrozen and its contents diluted with 4 mL of sterilized MilliQ-water to produce a 1 mM luciferin solution.

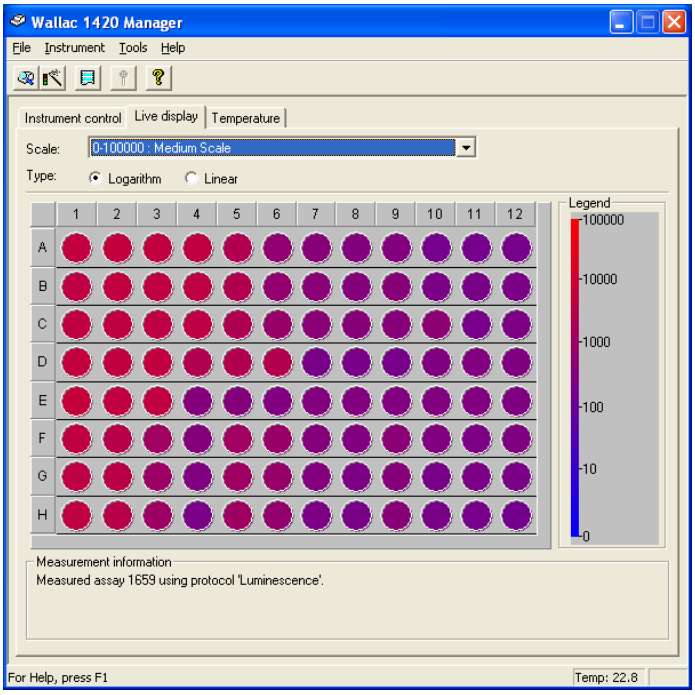

FIGURE 4.2. Standards for EE_2 and E_3, in addition with some spiked and real samples. The more red the color, the higher the yeast response to the estrogen.

In short, to construct a standard curve for all four estrogens (E_1, E_2, EE_2, E_3; **Table 4.3**), aliquots of estrogens (0.29–29 640 µg/L) were pipetted into white 96-well plates (**Paper II**). As blank, 10% EtOH was used. Aliquots of the yeast culture were subsequently added into the wells containing the urine samples and estrogen standards. Plates were incubated at 30°C for 2.5 h at 300 rpm on a rotary shaker. After incubation, aliquots of 1 mM $_D$-luciferin were pipetted into the wells and bioluminescence was measured using a Victor^2 Wallac 1420 Multilabel counter (**Figure 4.2**). In addition to signals from each well, the apparatus also indicated the intensity of the signal with colors.

The fold induction (FI) values were calculated based on signals given by the blank and the samples and compared to the estrogen standards to obtain approximation of estrogenic concentrations (**Paper II**). The effect of estrogen glucuronidation on bioavailability was tested by performing hydrolysis on the samples by heat-treating them in 80°C for 30 minutes or by adding β-glucuronidase enzyme and incubating at 46°C for 45 minutes to break the conjugated bonds.

4.3.3 Analytical Methods and Calculations

The analyses conducted during the experiments are summarized in **Table 4.5**. All standardized analyses were, in general, conducted using Finnish standards (SFS).

TABLE 4.5. Summary of the analyses used in this work.

Physicochemical, biological, and instrumental analysis	**Analytical method**	**Paper**
bioluminescence	luminometer	II
biomass concentration (dry weight, volatile suspended solids, VSS)	furnace, balance	IV
conductivity	electrode	II, III, IV
de-conjugation	β-glucuronidase addition, incubation	II
dissolved Na, Mg, K, Ca, Fe	atomic adsorption spectrophotometer (AAS): SFS 3044, SFS 3018	II, IV
dissolved organic carbon (DOC)	total organic carbon analyzer: SFS-EN 1484	II, III, IV
dissolved oxygen	electrode	IV
dissolved PO_4^{3-}, NO_2^-, NO_3^-, Cl^-, SO_4^{2-}	ion chromatography: SFS-EN ISO 10304-1	II, III, IV
estrogenic activity	yeast bioluminescence assay	II
filtration	vacuum or syringe filter with 0.2 or 0.45 µm membrane	I, III, IV
N_{tot}, P_{tot}, NH_4^+-N	colorimetric cuvette test, spectrophotometer	IV
optical density	spectrophotometer, @600 nm	II, IV
pH	pH-electrode	III, IV
pharmaceutical concentration	high performance liquid chromatography	I, III
sample preparation	solid phase extraction with vacuum manifold	I
temperature	electrode, data logger	III, IV
urea	ammonia selective electrode	II, III, IV
Microbiological analyses		
algal cell enumeration	microscopic counting with cell-counting chamber	IV

5 Results and Discussion

5.1 Urine Characteristics

The characteristics of urine used in different experiments (**Papers II–IV**) are described in **Table 5.1**. Urine was collected several times during years 2012–2014 from volunteers (see **Chapter 4.1.1**).

The pH of fresh urine was in the range of 5.7–6.3 (**Papers II–IV**) as reported in the literature and close to the average pH 6.0 of urine (Harper *et al.* 1979). As discussed in **Chapter 2.1**, the composition of human urine varies greatly with diet. This can explain the variation in different set-ups in e.g. urea concentration (0.89 g/L vs. 6.28 g/L in **Papers III** and **IV**, respectively) or in DOC (7.3 g/L in **Paper II** vs. 2.4 g/L in **Paper IV**), as well as with other parameters such as nutrients, anions, and cations. All in all, the nutrients and parameters in urine used (**Papers II–IV**) fall in the range reported in the literature (**Table 5.1**).

The pH of urine after storage (5–6 months) had risen well above 9 (**Papers II** and **III**), indicating ureolysis which is supported by the smaller concentration of urea in the stored urine as well as the increased ammonium, bicarbonate and alkalinity content of urine (**Table 5.1**). The decomposition of organic matter, precipitation of Mg and Ca salts (e.g. as struvite) and decrease in sulphate and phosphate content are supporting decomposition of urine into struvite during storage (**Papers II** and **III**). In general, during storage of five to six months, changes in conductivity followed the changes in pH. As pH rose, so did the conductivity, reaching up to 20–25 mS/cm (**Papers II** and **III**), while with lower final pH values the conductivity remained approximately at 10–13 mS/cm (**Paper III**). The increase in conductivity in stored urine is directly related to the amount of dissolved ions, e.g. phosphorus, nitrogen, and carbon: the higher the conductivity, the more there are ions present in the sample (Lind *et al.* 2001). This is in correlation with the present findings (**Papers II** and **III**), since dissolved nitrogen, phosphorus, and sulphur as well as cations were present throughout the storage period.

TABLE 5.1. Characteristics of the urine used in the experiments (**Papers II–IV**).

	Fresh	**Stored**	**Fresh**	**Stored**	**Fresh/ set-up I**	**Fresh/ set-up II**	**Range reported in the literature**
Parameter (unit)	*n = 6*		*n = 8*		*n = 1*		
pH	5.8	9.4	6.3	9.6	5.8	5.7	5.6–9.1
conductivity (mS/cm)	9.1	25.9	9.1	25.9	-	-	13.4–22.6
DOC (g/L)	7.29	2.81	3.9	1.5	4.83	2.405	-
$NH_4^+ + NH_3$ –N (g/L)	0.57	8.67	0.26	1.04	-	-	-
urea (g/L)	4.95	1.05	0.89	0.05	-	-	0.007–5.8
N_{tot} (g/L)	-	-	-	-	6.80	4.32	1.8–9.2
NO_3^--N (mg/L)	-	-	-	-	< 5	< 2	0–0.045
NO_2^--N (mg/L)	-	-	-	-	< 1	< 1	0–0.020
NH_4^+-N (mg/L)	-	-	-	-	520	275	254–1726
N_{org} (g/L) [a)]	-	-	-	-	6.275	4.042	-
P_{tot} (g/L)	-	-	-	-	0.670	0.355	-
Anions							
NO_3^- (g/L)	0.16	b.d.	0.0324	b.d.	-	-	-
NO_2^- (g/L)	-	-	0.04	0.77	-	-	-
SO_4^{2-} (g/L)	1.61	1.28	1.61	0.91	-	-	0.175–1.315 (as SO_4-S)
PO_4^{2-} (g/L)	2.86	1.18	0.93	0.35	-	-	0.076–2.0 (as P)
F^- (mg/L)	91.6	55.8	-	-	-	-	-
Cl^- (g/L)	2.67	2.40	-	-	-	-	1.4–6.62
Cations							
Na (g/L)	0.83	1.01	-	-	0.710	-	0.837–3.45
K (g/L)	0.52	1.17	-	-	0.717	-	0.770–2.74
Mg (mg/L)	76.0	b.d.	-	-	50	-	0–77
Ca (mg/L)	88.0	3.1	-	-	77	-	0–129
Fe (mg/L)[b)]	-	-	-	-	< 1	-	0.165–0.205
Reference	**II**	**II**	**III**	**III**	**IV**	**IV**	**Table 2.2**

Note: [a] N_{org} - urea; [b] below AAS detection limit; - not measured; b.d - below detection limit

5.2 Assessment of Analytical Methods for Urine Characterization

In this section, the applicability of two analytical methods on urine characterization are presented and discussed. Development of a new analytical tool and application of a previously published one on a new matrix provided important information on pharmaceutical and estrogenic compounds in urine during storage.

5.2.1 Applicability of the New HPLC Method in Pharmaceutical Detection in Urine (I)

As pharmaceutical analysis of complex matrices requires sample pre-treatment (Fontanals *et al*. 2007) and optimization of the analytical method for best recovery (Pavlović *et al.* 2007), the developed HPLC method provided a novel tool for fast analysis of selected pharmaceuticals. The compounds belonged to therapeutic groups which had different physicochemical properties (**Table 4.3**), which resulted in making compromises in the performance (Pavlović *et al.* 2007). As discussed earlier, simultaneous detection of different pharmaceuticals have been conducted (**Chapter 2.3.1**), but many of them have been dealing with compounds with similar properties, and prior to development of this method, no such tool existed that enabled simultaneous analysis of these eight compounds from one sample. Urine storage experiments

(**Paper III**) included 60 experimental jars (sampled monthly, **Chapter 4.2.1**), thus making it important to take into account the feasibility aspects of the method. Regarding future experiments, it was therefore crucial to enable the fast detection of the selected compounds.

Core-Shell Column Performance

However, by the time the method development was started, a new core-shell column technology was introduced in the analytical field. The column, which was used in this thesis (Kinetex XB-C_{18}, Phenomenex), utilized new 2.6 µm core-shell particles, which have a 1.9 µm, solid silica core and a 0.35 µm, thick porous silica layer on top, and which have been shown to perform well in gradient separations (Gritti and Guiochon 2010b) and under isocratic conditions (Gritti *et al.* 2010). Selection of the column was based on literature recommendations demonstrating the efficiency and performance of 2.6 µm core-shell columns to be similar or better than fully porous 1.7 µm particles in fast gradient separations (Fekete and Fekete 2011). The method development was performed according to recommendations given in the literature (Ruta *et al.* 2012) regarding the use of small system dwell volumes (the system volume was minimized e.g. with shorter capillary), ACN–water mixtures (ACN–water-based buffer mixture was used) and short column lengths (75x4.6 mm column).

The use of the core-shell technology provided both savings in time and money as the analysis time was reduced through application of larger backpressure of the column (>300 bar) and high flow rate (2.5 mL/min). As described in the literature (Gritti and Guiochon 2010b), operating the HPLC system with pressures under 400 bar was possible even with significantly higher mobile phase velocity (2.5 mL/min) than is recommended for porous particle columns. Although high flow rate causes faster use of the eluent, the reduced analysis time compensates for the increased mobile phase consumption. In a 6-minute sample run, a 15 mL mobile phase was consumed, as opposed to 18–21 mL with 1.5 mL/min flow rate and 12–14 minutes of analysis time, a starting point from which the method development was begun by using a fully porous 5 µm reversed-phase column. In addition, the new method was operated at 40°C as increasing the column temperature can cut down analysis time by reducing the mobile phase viscosity and thus increase mass transfer (Fekete *et al.* 2011). Based on method validation results, the best performance was achieved at this temperature.

Solid Phase Extraction Efficiency in Urine Pre-treatment

A sample pre-treatment was required due to the complex nature of the urine matrix (Escher *et al.* 2005). The SPE sorbent used was Strata-X (Phenomenex) which has been previously used e.g. in cleaning up biological samples (plasma, milk), and in enriching pesticides and pharmaceuticals from water samples (Fontanals *et al.* 2007 and references therein, Pavlović *et al.* 2007). Urine not pre-treated with SPE contains compounds that can interfere with the pharmaceutical analysis (**Figure 5.1**), especially with smaller concentrations. 3TC, TRI, CIP, and ZDV are the compounds which are most affected by analytics. Urine matrix interference especially that of a concentrated or hydrolyzed urine, has been reported previously to affect the identification of pharmaceuticals (Lai *et al.* 1997). Although in **Figure 5.1** the

pharmaceuticals are all visible, their signal intensity at 10 mg/L is quite different. This comes from the selected 210 nm wavelength used for signal recording. This particular wavelength has been used previously e.g. in screening a variety of toxicological drugs from serum and urine (Lai *et al.* 1997). Each compound has its own maximum intensity wavelength on which the observed signal would be the best. In order to optimize the analysis, 210 nm was used for all other compounds except for RMP, to which 264 nm was used.

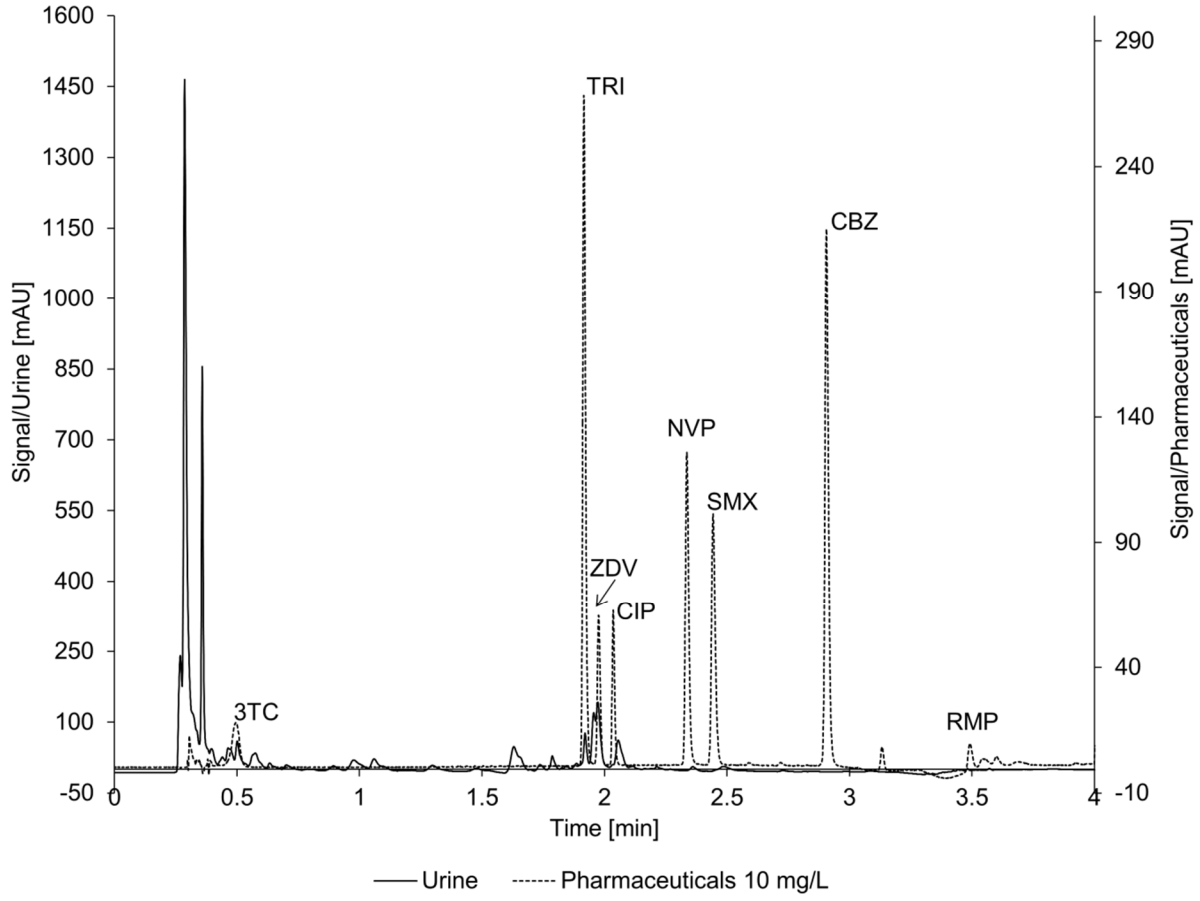

FIGURE 5.1. A graph showing HPLC-UV signal at 210 nm from filtrated urine (solid line) and pharmaceuticals (dashed line). Note the different y-axis scale for pharmaceuticals.

As the compounds studied were of varying physical and chemical properties, some compromises were needed to enable simultaneous analysis. With SPE, the limitations of the sorbent (Strata-X, $\log K_{ow}$ -0.5) affected the analysis of 3TC, which is why a two-step elution was needed. Antiviral drugs have been described as difficult to analyze because of the differences in their pK_a values and high polarity, and the fact that they are difficult to retain on common SPE sorbent materials (Prasse *et al.* 2010). SPE was used to clean up the samples and its use also provided savings, as the compounds could be eluted in two subsequent elutions from the same SPE tube, thus saving sorbent material. Comparison of different SPE sorbents on pharmaceutical recovery has been discussed in **Paper I**. 3TC performance was difficult to optimize in HPLC analysis as well, as it seemed that the linear range of the

compound was only up to 100 mg/L after which the compound was flushing out of the column with no retention. The injection of small enough sample sizes is therefore important to avoid overloading the column (Gritti and Guiochon 2012). The limited sample capacity is directly related to the core-shell particle surface area (DeStefano *et al.* 2012): Kinetex particles are 61% porous, which reduces the sample loading capacity due to the more straightforward movement of the sample in the column as a narrow band (McCalley 2011).

Method Suitability in Urine Sample Analysis with HPLC

The LOD (limit of detection) and LOQ (limit of quantification) of the method were somewhat high for urine samples. However, keeping in mind the further use of this method (**Paper III**), the LOD and LOQ obtained for the developed method were comparable with other similar studies with pharmaceutical detection from biological fluids and water (**Table 5.2**). As previously described, some compromises were needed for the method in order to be able to simultaneously analyze all eight compounds. Previously, e.g. antiviral pharmaceuticals have been analyzed simultaneously (Mistri *et al.* 2007), but these compounds all belonged to the same therapeutic group with relatively similar logK_{ow}'s (**Table 4.3**). In conclusion, the method performed as was expected, with acceptable LOD and LOQ, and after validation it was suitable to be used in further studies.

TABLE 5.2. Comparison of the limit of detection (LOD) and limit of quantification (LOQ) for pharmaceuticals from different matrices analyzed with HPLC.

Method	Matrix	Compound	LOD (µg/L)	LOQ (µg/L)	Sample pre-treatment	Recovery %	Reference
HPLC-UV	urine	3TC	1610	5366	SPE	16.7–43.3	**Paper I**
		ZDV	189	630	SPE	93.2–106.9	**Paper I**
		NVP	71	237	SPE	98.5–105.0	**Paper I**
		TRI	39	129	SPE	98.7–104.9	**Paper I**
		SMX	115	383	SPE	83.0–96.2	**Paper I**
		CIP	189	631	SPE	81.6–109.2	**Paper I**
		RMP	503	1676	SPE	90.7–105.8	**Paper I**
		CBZ	41	136	SPE	97.8–102.1	**Paper I**
		SMX	120	420	SPE	86.4–90.5	Fernandez-Torres *et al.* (2010)
		TRI	180	570	SPE	70.2–77.2	Fernandez-Torres *et al.* (2010)
HPLC-FL	serum	CIP	5	-	protein precipitation, enrichment	97.8–107.9	Zotou and Miltiadou (2002)
		CIP	50	-	sample vortexing, protein precipitation	99.4–101.0	Krol *et al.* (1995)
		CIP	50	-	sample dilution	99.0–104.3	Krol *et al.* (1995)
HPLC-MS/MS	water	TRI	0.00007	0.00025	SPE	46	Nebot *et al.* (2007)
		SMX	0.00013	0.00043	SPE	16	Nebot *et al.* (2007)
HPLC-MS	urine	CBZ	2400	-	un-treated	-	Maggs *et al.* (1997)
HPLC-MS/MS	plasma	3TC	25	-	SPE	87.6–90.9	Mistri *et al.* (2007)
		NVP	50	-	SPE	96.5–99.5	Mistri *et al.* (2007)

Note: - = not reported; UV - ultraviolet; FL - fluorescence

5.2.2 Efficiency of the Yeast Biosensor in Estrogenic Activity Assessment (II)

The goal of this study was to investigate the applicability of the bioluminescent yeast-cell-based biosensor as a fast, stand-alone analytical screening tool in estrogenic activity monitoring in urine. Urine samples required no pre-treatment (e.g. filtration, extraction, or centrifugation) and the method could be applied to urine as such.

Hormonal Activity Assay

The yeast biosensor produced a signal with respect to all of the studied estrogens (E_1, E_2, EE_2, E_3) in a concentration-dependent way (**Figure 5.2A**), similarly to Salste *et al.* (2007) who used the same biosensor to study WWTP effluents, thus demonstrating the repeatability of the results in a different laboratory and matrix. The yeast assay is specific to estrogenic substances. Studies have demonstrated that the estrogenic analysis with a yeast biosensor is not interfered by the presence of androgens or gestagen progesterone, and no interference is observed screening at low estrogen levels (Bovee *et al.* 2005, Salste *et al.* 2007). Mainly the response was obtained with estrogen range from 0.29 to 29 640 µg/L. The highest response with the biosensor was obtained with the synthetic EE_2. In order to estimate the overall estrogenic concentration, an average of four estrogens was taken (**Figure 5.2B**) to approximate concentrations in urine.

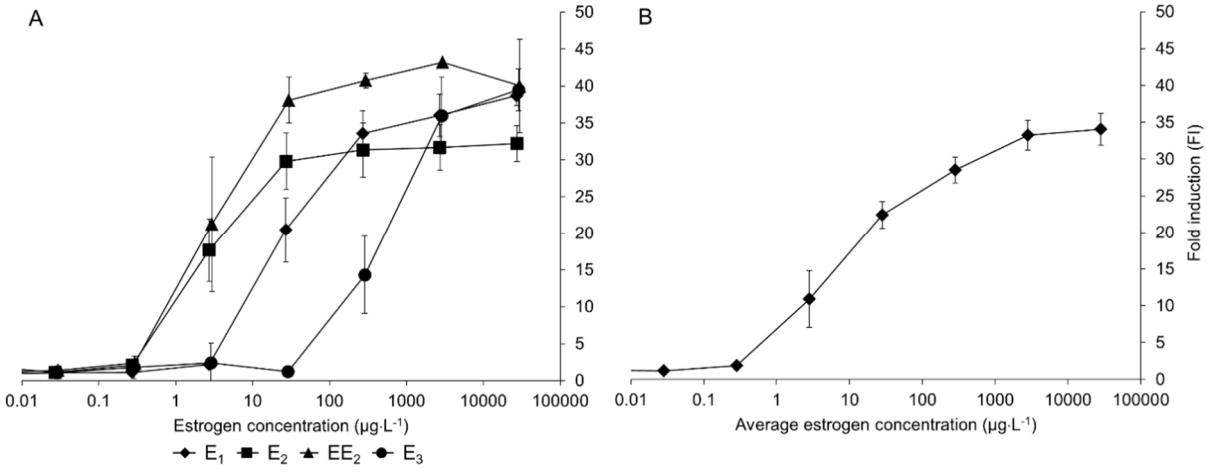

FIGURE 5.2. A) Standard curves for estrone (E_1), 17β-estradiol (E_2), 17α-ethinylestradiol (EE_2), and estriol (E_3). B) A standard curve calculated as the average of four estrogens from Figure 5.2A for the estimation of estrogenic concentration. The error bars represent the standard errors from three measurements. Estrogenic concentration determination: FI < FI_{LOD}: estimated from average estrogen curve; FI > FI_{LOD}: calculated from average estrogen curve; FI > average estrogen curve FI: calculated from EE_2 standard curve. (**Paper II**)

The estrogen LODs for the biosensor and the LOD determined from the average concentration curve were as presented in **Table 5.3**, in which a comparison of different yeast-cell-based bioassays and their detection limits for the current bioassay is also presented. Many studies have measured estrogen equivalents or used the bioassay only to demonstrate estrogenic

activity, and in those cases concentrations have been measured with chromatographic methods, requiring sample enrichment before analysis if the matrix has been some other than a standard solution (EtOH etc.). The LOD was practical in monitoring of real urine samples. Compared with the sample preparation and clean-up needed before chromatographic analysis (e.g. Escher *et al.* 2005), use of the biosensor was justifiable in simple and rapid monitoring. Compared with analysis using MS, which normally has a low LOD of only few ng/L (e.g. Desbrow *et al.* 1998), the obtained LODs were higher, but compared with conventional LC in combination with urine sample pre-treatment which has a LOD around 0.1–0.2 µg/L, the results are comparable (Zou *et al.* 2012). A comparison of different yeast-cell-based bioassays and their detection limits to chromatographic techniques is presented in **Table 5.4**.

Leskinen *et al.* (2005) have already proven that yeast estrogen strains have behaved and responded to hormones (two androgens, E_2) and xenoestrogens similarly with yeast estrogen screen. Urine always contains estrogens (even urine of males); thus, having urine blank is not possible. Stored men's urine has a low estrogenic background which is still higher than in fresh urine: this difference has been explained by the fact that majority of estrogens are excreted as conjugates which may be hydrolyzed in non-sterile urine storage (Escher *et al.* 2006). Even though urine stored long enough might be devoid of estrogens, the composition of the sample would be different and it might still contain estrogen-like compounds that induce a signal with the biosensor.

As discussed previously (**Chapter 4.1**), no filtration or sterilization of the urine samples was conducted as their costs would not be feasible in practice in a large scale – all the bacteria responsible for estrogenic degradation would be removed – and it was not considered significant related to the use of the biosensor in this thesis.

TABLE 5.3. Comparison of yeast-cell-based bioassays (**Paper II**).

Bioassay	**Reporter protein**	**Matrix**	**Sample pre-treatment**	**Time needed for analysis**	**Limit of detection LOD** (µg/L)	**Reference**
Saccharomyces cerevisiae BMAEREluc/ERα	luciferase	pure compound in 10% EtOH	not needed	2.5-h incubation	0.008 (E_2)[a]	Leskinen *et al.* (2005)
Saccharomyces cerevisiae (yEGFP)	green fluorescent protein	spiked calf urine	solid phase extraction, de-conjugation	de-conjugation overnight, plate drying overnight, 24-hour incubation	1.0 (E_2 and EE_2)[a]	Bovee *et al.* (2005)
Saccharomyces cerevisiae	β-galactosidase	pure compound in EtOH	not needed	three-day incubation	0.0015 (E_2)	Routledge and Sumpter (1996)
yeast estrogen screen (YES)	β-galactosidase	river water	solid phase extraction	pre-treatment 120-150+ min, three-day incubation	0.0001 estrogen equivalents (EE_2)	Vermeirssen *et al.* (2005)
Saccharomyces cerevisiae BMAEREluc/ERα	luciferase	wastewater effluent	solid phase extraction	solid phase extraction 20+ min, freeze drying 4.5 h, chromatographic fractionation 28 min, LC-MS/MS[b] 15 min, 2.5-h incubation	LOD was not determined for yeast assay	Salste *et al.* (2007)
Saccharomyces cerevisiae BMAEREluc/ERα	luciferase	pure compounds in 10% EtOH	not needed	2.5-h incubation	2.8 (E_1), 0.28 (E_2), 0.29 (EE_2), 35 (E_3)	**Paper II**
		human urine (real samples)	de-conjugation	de-conjugation 45 min, 2.5-h incubation	0.38–3804 (measured overall estrogenic concentration)	**Paper II**

Note: EtOH - ethanol; [a] measured as nM, converted by the authors; [b] LC-MS/MS - liquid chromatography-tandem mass spectrometry

TABLE 5.4. Comparison of the yeast biosensor detection limits with chromatographic techniques described in the literature.

Method	**Matrix**	**Compound**	**LOD** (µg/L)	**Sample pre-treatment**	**Recovery** (%)	**Reference**
HPLC-UV	water	E_1	330	SPE, enrichment	92	Stafiej *et al.* (2007)
		E_2	440		95	
		EE_2	510		91	
		E_3	240		75	
LC-MS/MS	wastewater effluent	E_1	0.0001	SPE, enrichment	105	Salste *et al.* (2007)
		E_2	0.0007		80	
		EE_2	0.001		43	
		E_3	0.005		80	
HPLC-UV	spiked human urine	E_1	0.2	cloud-point extraction	87.9–104.3	Zou *et al.* (2012)
		E_2	0.1		86.9–104.0	
Saccharomyces cerevisiae	fresh and stored human urine	overall estrogenic activity	0.38	de-conjugation	-	**Paper II**
	10% EtOH	E_1	2.8	none	-	**Paper II**
		E_2	0.28			
		EE_2	0.29			
		E_3	35			

Effect of Bioavailability of Estrogens in the Biosensor Application

The bioavailability of the estrogens and the response of the biosensor was affected by the presence of estrogens as conjugates in urine, thus breaking up the conjugated bonds improved the sensitivity of the sensor strain. Time points of one and five months were selected in estrogenic activity testing due to the availability of samples. The overall, recommended time for urine storage is six months (WHO 2006) but shorter periods are commonly used (e.g. Akpan-Idiok *et al.* 2012).

As most of the estrogens (85–95%) are excreted as glucuronides (**Table 2.7**), it was reasonable to assume that use of β-glucuronidase was sufficient to break the conjugate bonds. Of course, if one would want to be absolutely sure to get the more accurate estrogen concentration and better hydrolysis, the additional use of sulfatase would be justifiable. Breaking down conjugated bonds in urine is important to gain knowledge on the amount of estrogens, as some soil microorganisms are capable of breaking down these conjugates – thus releasing active hormones (Ying and Kookana 2005, Kurisu *et al.* 2010, Goeppert *et al.* 2014).

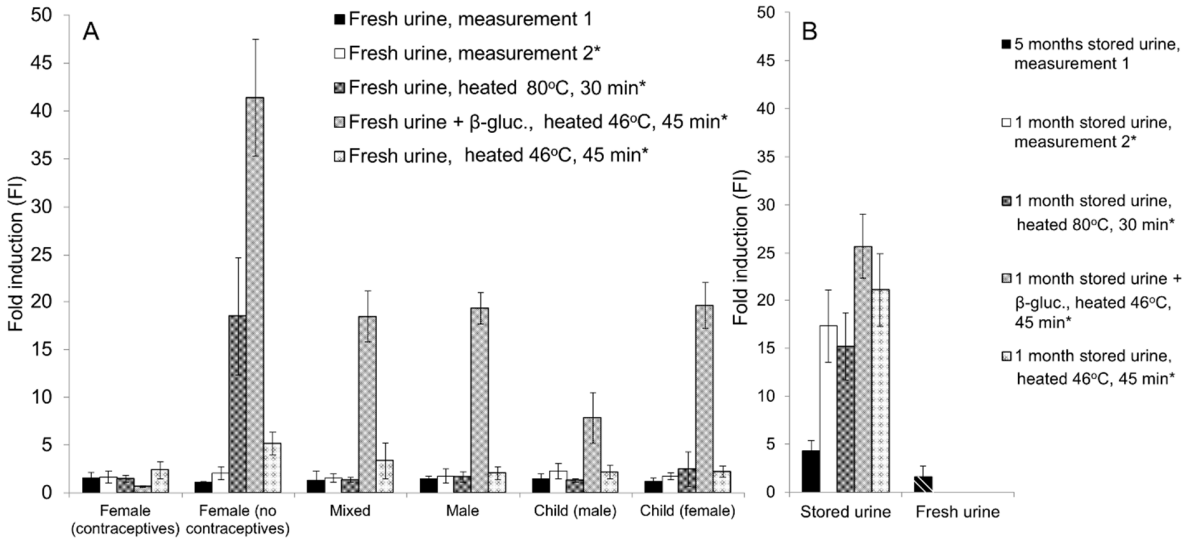

FIGURE 5.3. Fold inductions (FI) of different urine samples using the bioluminescent yeast biosensor. The bars represent mean of three replicates with error bars showing standard error. β-gluc. = β-glucuronidase. A) FI values for the different fresh urine samples with and without different pre-treatments. B) FI values of fresh and stored urine (at room temperature in plastic jars with a tight lid, one or five months) with and without different pre-treatments. * indicates that urine samples were collected and analysed at the same time (measurement 2). (**Paper II**)

Only unconjugated forms of estrogens and estrogenic compounds contributed to the ER activity when urine was measured without treatment (first and second bar, **Figure 5.3A**). Thus, the

bioavailability testing was conducted to get the response for all estrogenic activity present in the sample (measuring the sum of free and glucuronidated forms). Heat treatment at 80°C for half an hour resulted in increased activities (0.35–14 µg/L) when compared with urine without heating (0.05–0.29 µg/L). The addition of β-glucuronidase and incubation at 46°C for 45 minutes increased the activity (1.4–10 200 µg/L, determined using standard for EE_2), while incubation at 46 °C did not affect the activity as much as enzyme addition (**Figures 5.3A** and **5.3B**). Clearly, the method sensitivity was better with the β-glucuronidase addition, which was in accordance with previous results from testing a yeast biosensor with swine urine with pre-treatment before analysis to break the conjugated bonds (incubation with a *Helix pomatia* solution) (Kaiser *et al.* 2010).

The effect of urine storage on estrogenic activity was as speculated: the estrogenic activity in urine samples first increased from the 0.1 µg/L (< LOD) detected from fresh urine up to 9.5 µg/L after one month of storage. This was expected since source-separated urine is never devoid of bacteria. The bacterial enzymatic (glucuronidases, estradiol dehydrogenases and sulfatases) and metabolic activity break the conjugated bonds, thus increasing the observed signal which reduces over time due to estrogen degradation. After five months of storage the estrogenic activity was still 6.2 µg/L, indicating the presence of ER binding compounds.

Advantage of Yeast-Cell-based Biosensor over Chromatographic Techniques in Urine Analysis

No adaptations of the biosensor for urine matrix were needed, which emphasized the applicability of the biosensor without pre-treatments on different matrices. Biological analysis can be advantageous over chemical analysis since the concentrations of single compounds are quite low and trace analytical techniques would have to be developed to detect only a fraction of pharmaceuticals and hormones present (Escher *et al.* 2006). Although the measurement of exact concentrations in urine is informative, measuring the overall estrogenic activity can be more useful regarding practical applications, such as fertilizer use. The biosensor measures the cumulative concentration of EDCs in urine, not just estrogens. Furthermore, the assay provides reasonable information on estrogenic activity in order to state when the activity has been reduced adequately in order to re-use urine, as urine application has been shown to enhance leaching and persistence of EDCs in the soil (study conducted with sheep urine, Lucas and Jones 2009). The yeast biosensor would not replace any standard methods, but could be used by authorities to screen a large number of samples for preliminary indications (Rodriguez-Mozaz *et al.* 2007).

Biosensors are also suitable for the development for on-site tests because of their high specificity. Since estrogens have been shown to migrate and degrade in the soil (Ying and

Kookana 2005), this screening tool could be used to test estrogenic activity in soil samples after extraction to liquid. The yeast could also be used in pre-screening anthropogenic estrogen-like compounds which interact with the receptor, such as bisphenol-A or parabens (e.g. Leskinen *et al.* 2005). On the contrary to conventional chromatographic methods, the method has several advantages, including complementarity to traditional analytical chemistry analyses, a minimal sample pre-treatment need, the provision of analyte bioavailability information, and simultaneous analysis of multiple samples. The efficiency of the applied biosensor is due to the fast retrieval of results: compared to previous analyses which may take several days or include extensive sample pre-treatment (e.g. Routledge and Sumpter 1996), this yeast biosensor gave results in just three hours.

5.3 Effect of Urine Storage on Pharmaceuticals (III)

Behavior of eight pharmaceutical compounds during six-month storage of urine spiked individually, in therapeutic groups (antivirals, antibiotics, and anti-tuberculotics), and with different amendments (feces and urease inhibitor) was studied using CBZ as a reference pharmaceutical. In all assays, precipitates – either solid or both solid and floating – were visible at the bottom of the jars after six months, and in some cases already earlier. Only the liquid phase of the experimental jars was assayed: no precipitates were taken under examination.

pH during Urine Storage in Pharmaceutical Amendments

pH, which was followed as an indicator of biological and ureolytic activity, rose in jars where pharmaceuticals where applied individually to 8.7–9.6, whereas in therapeutic groups only anti-tuberculotics had lower pH (7.9) (**Table 5.5**). This was expected to be due to the inhibitory effect of CIP and RMP on bacteria present in urine. Adding feces resulted in lower pH in the groups of antibiotics and anti-tuberculotics. The assays with urease inhibitor amendment resulted in the lowest final pH of 7.4–8.2 (**Table 5.5**), which was in correlation with the urease inhibitor delaying urea hydrolysis (Watson *et al.* 2008). In fact, pH of un-amended urine was >9 after two months, whereas pH with urease inhibitor remained <7 for the first three months.

Effect of Storage on Pharmaceutical Concentrations

The overall pharmaceutical concentration reductions in the liquid phase without amendments were 41.9–99% for anti-tuberculotics, <52% for antivirals (except 3TC 75.6%), and <50% for antibiotic compounds. In assays with amendments, concentrations reductions remained <50%, except for RMP (>99%). RMP was completely removed in all assays (**Table 5.6, Figure 5.4**), indicating that the precipitation was not the major removal mechanism, but probably

biodegradation, which would be a novel finding as no information of RMP biodegradation could be found e.g. from WWTPs (**Table 2.9**). Co-precipitation of CBZ and other pharmaceuticals with struvite is not very likely as, using mass balances, it has been shown that pharmaceuticals remain in the solution (> 96% of CBZ, Ronteltap *et al.* 2007).

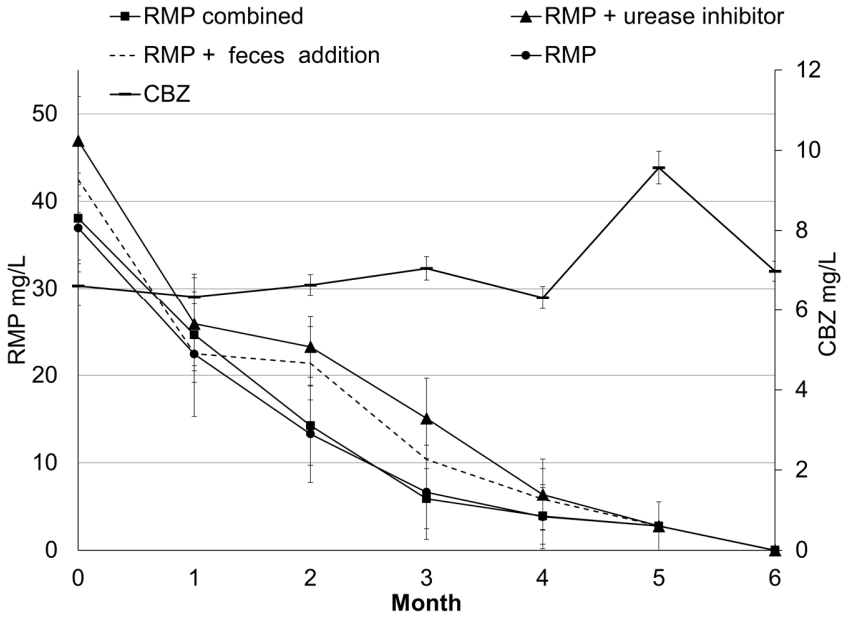

FIGURE 5.4. The concentration of rifampicin (RMP, left y-axis) and carbamazepine (CBZ, right y-axis) in monthly samples during the six-month storage of urine with different amendments. Combined refers to the results of urine amended with therapeutic groups. The error bars represent the standard error between three replicates. (**Paper III**)

In addition to bacteria not being able to break down some of the pharmaceuticals entering WWTPs (see e.g. **Table 2.9** for WWTP removal percentages), transformation products are formed, which together with pharmaceuticals end up in the environment (Gao *et al.* 2012). The HPLC-UV chromatograms of monthly samples showed CIP and SMX producing a peak with almost similar spectrum to the parent compound, suggesting degradation (**Figure 5.5**). A qualitative LC-ESI-MS/MS (LC-electrospray ionization-MS/MS) applied on assays of individual compounds after a six-month storage showed no marked differences between different amendments. The removal of RMP was confirmed while four unidentified transformation products were observed in RMP assays. In addition, various transformation products for CIP, SMX, and 3TC were identified, while no transformation products for NVP, ZDV, and TRI (available in literature) were detected.

TABLE 5.5. pH after six months of storage. Data are mean (± standard error), n = 3. The different background colors represent the subdivision into therapeutic groups. (Modified from **Paper III**)

		pH							
		Separately		**Therapeutic groups**		**Feces**		**Urease inhibitor**	
		Start	6 months	Start	6 months	Start	6 months	Start	6 months
antivirals	3TC	6.3 (0.0)	9.3 (0.2)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	8.2 (0.1)
	ZDV	6.3 (0.0)	9.6 (0.0)						
	NVP	6.3 (0.0)	9.5 (0.0)						
antibiotics	TRI	6.4 (0.0)	8.7 (0.4)	6.4 (0.0)	9.2 (0.2)	6.2 (0.0)	7.8 (0.8)	6.5 (0.0)	7.4 (0.0)
	SMX	6.3 (0.0)	9.4 (0.2)						
anti-tuberculotics	CIP	6.3 (0.0)	8.9 (0.1)	6.4 (0.0)	7.9 (0.2)	6.2 (0.0)	7.4 (0.2)	6.5 (0.0)	7.7 (0.5)
	RMP	6.3 (0.0)	9.4 (0.1)						
reference	CBZ	6.4 (0.0)	9.6 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Control	6.3 (0.0)	9.6 (0.0)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	7.7 (0.0)

Note: n.a. - not available

TABLE 5.6. Reduction of pharmaceuticals in liquid phase after six months of storage. Data are mean (± standard error), n = 3. The different background colors represent the subdivision into therapeutic groups. “Individually” refers to just one pharmaceutical amended in urine. (Modified from **Paper III**)

		Reduction (%) in the liquid phase after six months				**Transformation products detected**	
	Pharmaceutical	**Individually**	**Therapeutic groups**	**Feces**	**Urease inhibitor**	**HPLC-UV**	**LC-ESI-MS/MS**
antivirals	3TC	75.6 (7.8)	51.4 (8.3)	28.9 (22.3)	< 1	n.d.	+
	ZDV	51.5 (3.7)	45.6 (0.5)	< 1	< 1	n.d.	n.d.
	NVP	25.6 (6.2)	28.8 (3.1)	24.5 (2.9)	16.9 (5.0)	n.d.	n.d.
antibiotics	TRI	23.7 (1.7)	40.3 (4.8)	42.0 (3.6)	18.9 (1.6)	n.d.	n.d.
	SMX	24.0 (4.7)	32.2 (3.0)	< 1	< 1	+	+
anti-tuberculotics	CIP	51.1 (10.6)	41.9 (27.4)	38.5 (8.5)	44.2 (19.5)	+	+
	RMP	> 99	> 99	> 99	> 99	n.d.	+
reference	CBZ	26.8 (3.5)	n.a.	n.a.	n.a.	n.d.	+

Note: n.a. - not available; n.d. - not detected; + - transformation product(s) detected

The emergence of breakdown products in LC and LC-ESI-MS/MS analysis suggested (bio)transformation and implied paired compounds forming into potentially more harmful products; the environmental relevance of transformation products is still quite a new field of study, but the possibly higher risk of transformation products compared with the parent compound on human health and the environment is a fact (Haddad *et al.* 2015). However, chemical degradation also produces transformation products, which cannot be ruled out.

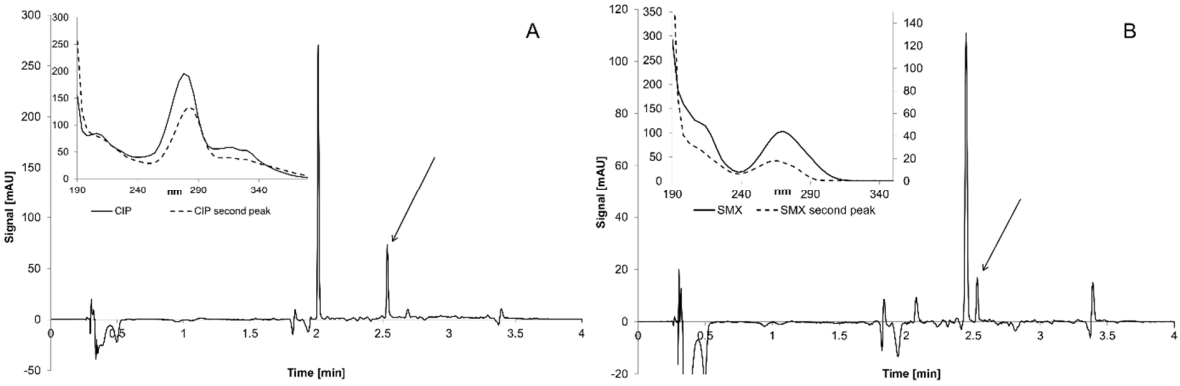

FIGURE 5.5. Chromatograms and spectra of CIP (A) and SMX (B) after five months of storage of urine amended with individual pharmaceuticals. The additional peaks are indicated with arrows. The spectra of the additional peaks resemble the original compounds. Note that the signal of SMX second peak is on the secondary axis. (**Paper III**)

Biological removal was suggested by the disappearance of RMP parent compound and appearance of transformation products in LC-ESI-MS/MS analysis, but the degradation could also be chemically induced. The complete removal of RMP in each assay was strikingly opposite to the concentrations of the other seven studied pharmaceuticals which were reduced only moderately. The degradability rate of RMP, and partly SMX and CIP, may have increased with higher bacterial densities, and the diversity of microorganisms in urine could have increasingly affected the biodegradability as source-separated urine can support the growth of bacteria up to about 10^8 cfu (colony forming unit)/mL (Brooks and Keevil 1997). In comparison, closed bottle tests, which are common in biodegradation analysis, use low bacterial densities of 10^4–10^6 cfu/L (OECD 1992). On one hand, the pH increase suggested biological activity, but on the other, similar removals during storage were observed without pH change.

Based on the findings, six-month storage of urine reduces the pharmaceutical concentrations (23–75%), while storage may result in the formation of transformation products – suggesting that storage as recommended by WHO does not mean complete removal of the studied pharmaceutical risks. One has to bear in mind though that the results only apply to the eight pharmaceuticals and concentrations, which were quite high to replicate a worst-case scenario

of almost all people eating pharmaceuticals. If urine is to be utilized in fertilizer applications, other feasible treatment practices to enhance pharmaceutical removal should be considered. Some urine treatment processes and the removal of pharmaceuticals and hormones were presented in **Chapter 2.2.2**, but as discussed earlier, the techniques can be somewhat energy-intensive.

The presence of pharmaceuticals in separately collected urine and the possible degradation of pharmaceuticals has been speculated previously. Urine from a male urinal stored for several weeks contained many pharmaceuticals (e.g. lipid regulators and painkillers), but the measured concentrations were lower than calculated ones, suggesting that degradation processes might occur on some pharmaceuticals during storage (Winker *et al.* 2008b). Only few investigations on pharmaceutical behavior in source-separated urine have been performed: some in stored urine (Gajurel *et al.* 2007, Tettenborn *et al.* 2007) and some during precipitation of struvite (Ronteltap *et al.* 2007, Keemacheevakul *et al.* 2012). Most of them were focusing on lipid modifiers, painkillers, etc., while little or no data on fate of the compounds selected in this thesis was available. Based on the before-mentioned studies, however, it can be concluded that at least CBZ and TRI are not co-precipitating with struvite.

Storage was conducted in similar conditions of 20°C in the dark. Summary of the storage results and comparison of the removal percentages of different pharmaceuticals is given in **Table 5.7**. The compounds tested in the experiments presented in **Table 5.7** (except for CBZ) were different to aforementioned studies, as well as the formulation which was used to spike urine samples. Schürmann *et al.* (2012) used tablet formulations where the sample also contained additives such as lactose or starch, whereas only the pure compound was used in this thesis.

TABLE 5.7. Effect of urine storage on pharmaceutical concentrations in different urine storage experiments reported in the literature.

Pharmaceutical	Specification	Reduction	Reference
Bisoprolol	beta blocker	19.3–38.3%	Schürmann *et al.* (2012)
Chloroquine	prevention of malaria	14.3–71.6%	Schürmann *et al.* (2012)
Diclofenac	anti-inflammatory	22.1–97.3%	Schürmann *et al.* (2012)
		80–90%[a]	Butzen *et al.* (2005)
Tetracyclin	antibiotic	80–90%[a]	Butzen *et al.* (2005)
		40%[b]	Butzen *et al.* (2005)
Fenoprofen	anti-inflammatory	20%[b]	Butzen *et al.* (2005)
Hydrochlorothiazide	anti-hypertensive	-	Schürmann *et al.* (2012)
Ibuprofen	anti-inflammatory	-3.9–66.7%	Schürmann *et al.* (2012)
Metoprolol	beta blocker	27.3–77.5%	Schürmann *et al.* (2012)
Nebivolol	beta blocker	-	Schürmann *et al.* (2012)
Sulfadimidine	veterinary antibiotic	59.1–94.3%	Schürmann *et al.* (2012)
Tramadolol	opioid analgetic	20.9–57.7%	Schürmann *et al.* (2012)
Carbamazepine	anti-convulsant	24.4–79.8%	Schürmann *et al.* (2012)
		22.2–26.8%	**Paper III**
		~20%	Gajurel *et al.* (2007)
		20%	Butzen *et al.* (2005)
Lamivudine	antiviral	<1–71.6%	**Paper III**
Zidovudine	antiviral	<1–51.5%	**Paper III**
Nevirapine	antiviral	16.9–28.8%	**Paper III**
Sulfamethoxazole	antibiotic	<1–32.2%	**Paper III**
		45%[a]	Butzen *et al.* (2005)
		30%[b]	Butzen *et al.* (2005)
Trimethoprim	antibiotic	18.9–42.0%	**Paper III**
Ciprofloxacin	anti-tuberculotic	15.5–51.1%	**Paper III**
Rifampicin	anti-tuberculotic	>99%	**Paper III**

Note: Schürmann *et al.* (2012) results collected from a Figure representing elimination in pH-adjusted urine. [a] pH 2; [b] pH 9

During storage period of a year, none of the tested pharmaceuticals (**Table 5.7**) were substantially removed in spiked urine (Gajurel *et al.* 2007). Although different pH's (3, 4, 6.5, 7, 8.5, 9, 9.5, 10, and 11: Butzen *et al.* 2005, Gajurel *et al.* 2007, Schürmann *et al.* 2012), storage temperatures (4 °C, 20°C, room temperature varying between 12 and 38°C, Gajurel *et al.* 2007, Schürmann *et al.* 2012), and storage periods (six months, Schürmann *et al.* 2012; a year, Gajurel *et al.* 2007) have been reported, no marked effect on pharmaceutical concentrations have been discovered. Although the concentrations used in the storage experiment were relatively high, they were in the same order of magnitude as in the experiment of Gajurel *et al.* (2007), who spiked their urine with 10 mg/L of pharmaceuticals. When the results from storage experiments (**Table 5.6**) are compared with the pharmaceutical concentration reductions in urine treatment processes (**Table 2.8**), it is obvious that storage alone is not as effective as the treatment techniques.

Controversially, an earlier study (Butzen *et al.* 2005) has shown that low pH has a reducing impact on pharmaceutical concentrations during urine storage. After storage period of 3–4

months at pH 2, some compounds had reduced concentrations of 80–90%. It was acknowledged that the length of the storage period was not enough to remove these compounds, and the highest removal was achieved in acidic urine (Butzen *et al.* 2005). However, as discussed earlier (**Table 5.1**), pH of stored urine is elevated up to pH 9.6 – being far from acidic – and the costs of acidification would probably be too high in large scale applications. The prevention of urine hydrolysis, e.g. with urease inhibitors, is more economical than subsequent neutralization; e.g. hydrolyzed urine requires 11.3 g concentrated sulphuric acid per liter of urine (Maurer *et al.* 2006).

Effect of Feces Presence on Pharmaceutical Behavior during Urine Storage

The results clearly showed that with feces, the pharmaceutical concentration reductions were smaller than in therapeutic groups without additional amendments (<1–42% vs. 28–51%, respectively). Feces amendment did not enhance pharmaceutical removal, even though it was expected to increase microbial content in assay jars. This was interesting, as fecal contamination is prone to be present in collected urine but it had negative effect on pharmaceutical removal. Feces were added in urine samples containing pharmaceuticals in therapeutic groups to evaluate the effect of microorganism inoculum in pharmaceutical concentrations. The added concentration was 9 mg_{feces}/L, which has been found to be an average in source-separated urine (Schönning *et al.* 2002). It was hypothesized that the bacteria derived from feces would enhance biological removal of the compounds. Thus, the results imply that pharmaceutical removal may in fact be reduced in the presence of fecal contamination, but the mechanism is yet to be discovered and needs more research. Fecal microbes are inactivated at pH over 8.9 (Höglund *et al.* 2002), which could have affected the results.

Effect of Urease Inhibitor Presence on Pharmaceutical Behavior during Urine Storage

It appeared that the used urease inhibitor (2 mM, Tampere University of Technology *et al.* 2011) resulted in a low pH increase as anticipated, but however, it did not affect positively on pharmaceutical reduction. In fact, the pharmaceutical removal was lower in urine amended with urease inhibitor (<1–18.9%) than in control assays consisting of urine and pharmaceuticals (28.8–51.4%), except in the case of CIP (44.2%). Originally, the hypothesis was that lower pH generally enables better microorganism growth, thus enhancing biological removal of pharmaceuticals as bacterial extracellular enzymes can break down bonds in pharmaceutical molecules. Chemical precipitation/sorption of the studied pharmaceuticals was ruled out, as pH did not rise above 8, which is considered as a minimum pH prerequisite for the formation of struvite precipitates (pH optimum 9.4–9.7, Harada *et al.* 2006). The mechanism in pharmaceutical concentration reduction could well be biological and was inhibited by urease

inhibitor, leading to deduction that urease inhibitors could actually have negative effects on urine microbes and their functionality. However, contrary to this, in a greenhouse experiment where nBPT was applied in the soil, the soil urease activity was initially inhibited but recovered within 10 days (Watson 1998 and references therein).

HPLC analysis (**Paper I**) demonstrated that 59% of the applied urease inhibitor was still present after six months of urine storage. It has been hypothesized that the degradation products of fertilizer formulations might have an inhibitory effect on soil ureases (Watson *et al.* 2008) and while the height of nBPT peak in HPLC chromatogram fell in time, additional peaks appeared. The addition of urease inhibitor might have co-affected the removal of compounds by preventing enzyme activity, thus explaining the poor concentration reductions.

5.4 Microalgal and Biomass Growth in Human Urine (IV)

Microalgal and Biomass Growth

The use of diluted human urine as a sole nutrient source to grow biomass from microalga *Chlorella vulgaris* was studied using batch cultivations (21 days) in five different urine dilutions (1:25–1:300), in 1:100-diluted urine as such and with added trace elements (urine+TE), and as a reference, in artificial growth medium. First, the growth of *C. vulgaris* in 1:100-diluted urine was compared with growth in modified artificial Chu-10 medium and in Chu-10 medium where nitrogen and phosphorus sources were replaced with a corresponding amount of urine (set-up I) to investigate the potential of diluted urine as an algal nutrient source. Afterwards, the effect of different urine dilutions (1:25–1:300) on algal growth was assessed (set-up II).

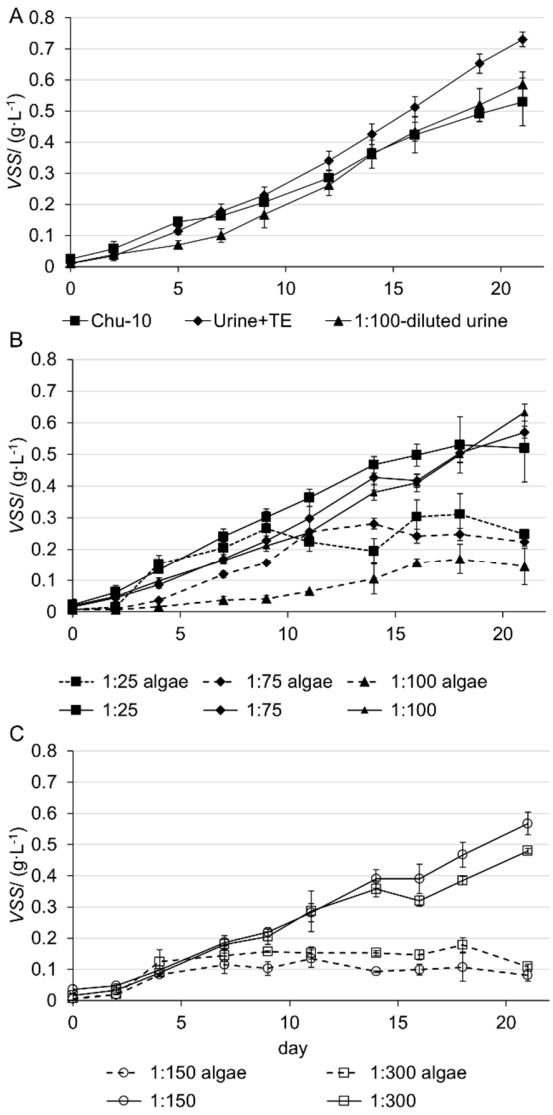

FIGURE 5.6. Biomass production as volatile suspended solids (VSS) in A) set-up I using 1:100-diluted urine, urine+TE and Chu-10, and as VSS and calculated VSS_{algae} B) in set-up II with urine dilutions 1:25, 1:75 and 1:100, and C) urine dilutions 1:150 and 1:300. The error bars represent the standard error of three (A) and two (B, C) replicates. (**Paper IV**)

C. vulgaris and related biomass grew better in urine than in the artificial medium (0.59 vs. 0.53 g/L) (**Figures 5.6A-C**, **Table 5.8**). As biomass yields in 1:100-diluted urine were higher than in modified Chu-10 medium, the next step was to test the effect of different urine concentrations (set-up II). It was discovered that after 5–10 days, the algal biomass stabilized to approximately 0.3 g/L, whereas total biomass continued to grow with similar growth rates and final biomass concentrations (0.5–0.6 gVSS/L) for all urine dilutions as well as for all reference cultures

(**Table 5.8**). Living algae dominated the biomass for the first 5–15 days – contributing about 50–100% of the biomass (in 1:100-dilution < 30%) – after which the concentration of living algal cells stabilized, being at the end of the incubations (day 21) less than 40% of the VSS in all dilutions (**Figures 5.6B** and **5.6C**). The high share of VSS_{algae} in the beginning of the cultivation suggested that in the beginning the conditions in all urine dilutions favored microalgal growth, while in the course of incubation bacteria became more competitive and the portion of dead algal cells increased. The obtained final biomass concentrations were in line with other results reported in the literature (**Table 5.8**), though not especially high. The growth in 1:100-diluted urine was, compared with other dilutions, unexpectedly poor in both replicates in set-up II, which could not be explained.

The biomass growth was likely not limited by nutrients but rather light or CO_2, as the 1:300 and 1:25-dilutions resulted in similar final VSS concentrations (0.48 vs. 0.52 gVSS/L). The light intensity used was quite low (29 µmol photons/m^2/s), and additional CO_2/air sparging could have improved the results as cultures might have lacked sufficient light due to the increasing biomass concentration, as mutual shading of algae in batch cultivation decreases the average light supply per microalgae cell (Tuantet *et al*. 2014a). However, *C. vulgaris* has been cultivated previously at similarly low light intensities of 25–30 µmol photons/m^2/s (de-Bashan *et al.* 2005, Moed *et al.* 2015).

Availability of trace elements (e.g. iron, magnesium) is considered a potential limiting factor when cultivating algae with urine. (Liu *et al.* 2008, Sydney *et al.* 2010, Ilavarasi *et al.* 2011, Tuantet *et al.* 2014a, 2014b). The urine+TE medium also contained added vitamins, magnesium, and iron, whereas undiluted urine contained more magnesium and approximately the same concentration of total iron as Chu-10. Hydrolysis of urine leads to precipitation and depletion of Mg, thus decreasing its availability (Tuantet *et al.* 2014a), which is why the use of urease inhibitors for urine preservation has been suggested (**Chapter 2.1.2**). However, dilution of urine and thus decreased trace element concentrations did not affect overall biomass growth. The lower concentration of trace elements may have led to a bacterial dominance in the cultures because there may not have been enough magnesium or iron to support the growth of photosynthetic algae which require these elements e.g. for the production of chlorophyll and photosynthetic enzymes. However, it was demonstrated that urine without any additional vitamins or trace elements can sustain microalgal growth.

TABLE 5.8. Comparison of different culture media and urine dilutions for algal growth in studies reported in the literature. Values given are averages.

	Cultivation set-up	Nitrogen source	Growth rate μ (1/d)	Biomass production (g/L)	Biomass productivity g/(L·d)	Reference
Algae grown in urine						
Arthrospira platensis (*Spirulina platensis*)	bubble column photobioreactor nitrification CO_2 addition 2000 μmol photons/m^2/s [a] 16-day cultivation	1:10-diluted synthetic urine 1:10-diluted urine	- -	2.5 2.30	0.237 0.206	Feng *et al.* (2007)
Spirulina platensis	batch culture, 12 days continuous culture, 28 days	synthetic urine	- -	1.25 2.9–3.4	- -	Yang *et al.* (2008)
Spirulina platensis	bubble column photobioreactor 5.6 L, nitrification 2000 μmol photons/m^2/s [a]	1:10-diluted urine	-	2.40	-	Feng *et al.* (2008)
Spirulina platensis	photobioreactor CO_2 addition	1:120-diluted synthetic urine 1:120-diluted urine	0.171 0.235	0.25 0.81	- -	Chang *et al.* (2013)
Spirulina platensis	bubble column photobioreactor 1.2 L, aeration 2000 μmol photons/m^2/s [a] 14-day cultivation	1:180-diluted urine 1:180-diluted synthetic urine	- -	2.32 2.4	0.266 0.236	Feng and Wu (2006)
Scenedesmus acuminatus	114-day cultivation, tank-scale 30-day cultivation, tank-scale	1:50-diluted urine	- -	0.133 [b] 0.039 [b]	- -	Adamsson (2000)
Chlorella sorokiniana	CO_2 addition photobioreactor controlled light intensity 490–1550 μmol photons/m^2/s	1:2-diluted synthetic urine 1:2-diluted urine	- -	8.1 6.6	7.5 9.3	Tuantet *et al.* (2014b)
Chlorella sorokiniana	24-well microtiterplate 105 μmol photons/m^2/s	concentrated urine synthetic urine	0.104[c] 0.116[c]	-	-	Tuantet *et al.* (2014a)
Chlorella sorokiniana	7 daily cycles 6 L photobioreactor 133 μmol photons/m^2/s CO_2 addition, pH adjustment	non-filtered fresh urine	1.18	-	-	Zhang *et al.* (2014)
Chlorella vulgaris	21-day cultivation batch bottles 29 μmol photons/m^2/s	1:25-diluted urine 1:75 1:100 1:150 1:300	0.38 0.34 0.20 0.35 0.38	0.520 0.570 0.600 0.570 0.480	0.06 0.07 0.07 0.06 0.05	**Paper IV**

TABLE 5.8. Continues

	Cultivation set-up	Nitrogen source	Growth rate μ (1/d)	Biomass production (g/L)	Biomass productivity g/(L·d)	Reference
Chlorella vulgaris grown in growth medium						
Chlorella vulgaris	CO_2 addition 14-day cultivation Photobioreactor 225 μmol photons/m^2/s	M8 (urea)[d]	-	0.53	0.052	Hulatt *et al.* (2012)
Chlorella vulgaris	CO_2 addition 14-day cultivation photobioreactor 225 μmol photons/m^2/s	M8 (nitrate)[d]	-	0.54	0.046	Hulatt *et al.* (2012)
Chlorella sp.	1 L photobioreactor CO_2 addition 600 μmol photons/m^2/s	Walne's medium 0.1 g/L urea	0.058[c]	1.422	-	Hsieh and Wu (2009)
Chlorella vulgaris	12-day cultivation initial pH 5 60–70 μmol photons/m^2/s	organic fertilizer	0.270	0.51	-	Lam and Lee (2012b)
Chlorella vulgaris	21-day cultivation shake flasks 29 μmol photons/m^2/s	Chu-10 (mod.) urine+TE	0.370 0.310	0.529 0.730	0.056 0.080	**Paper IV**

Note: - not reported; [a] 444.4 W/m^2 converted to μmol photons/m^2/s using conversion factor of 1 W/m^2 = 4.5 μmol photons/m^2/s; [b] calculated from cells/mL with a conversion factor by the authors; [c] growth rate reported as (1/h); [d] nitrogen limitation (1.33 mM)

Bacterial presence in the cultivations enhanced *C. vulgaris* flocculation, and clump formation in the presence of bacteria and their extracellular polymeric substances has been previously reported, although algal cells have naturally a negative charge on the cell surface which prevents aggregation (Lee *et al.* 2013). Non-sterile cultivation conditions are beneficial for nutrient removal over sterilized growth conditions (*Chlorella* sp. grown on secondary effluent, Zhang and Hong 2014) and bacterial (*Bacillus licheniformis)* presence in *C. vulgaris* cultivation has led to better ammonium and total phosphorus (P_{tot}) removal efficiency than in single algae or bacteria system in addition to promoting algal growth (Liang *et al.* 2013).

The source for bacteria in algal cultivations was obvious as urine contains bacteria, the presence of which was microscopically confirmed in this study, and which in fact can be expected as urine contains organic carbon (**Table 5.1**) which enables growth of competitive microorganisms such as bacteria (Perez-Garcia *et al*. 2011a). The bacterial presence in sterilized and non-sterilized artificial algal growth media has been reported previously (Lakaniemi *et al.* 2012a, 2012b), but as urine contains bacteria, it was thought it might have a major impact on algal growth. Only two studies investigating the possible presence of bacteria in algal cultivations on urine have been conducted previously; *C. sorokiniana* grown with undiluted urine revealed heterotrophic bacteria growth in microscopical inspection (Zhang *et al.* 2014), while bacterial presence has also been estimated via chemical oxygen demand (COD) depletion (Tuantet *et al.* 2014b).

pH and DOC Concentration during Cultivations

Biomass growth was similar during the cultivations (**Figures 5.7A** and **5.7B**) even though pH varied greatly between different cultivation batches (pH range 4.5 to 10). In urine dilutions 1:25–1:100 the pH increased after an initial drop (**Figure 5.7B**) apparently due to CO_2 consumption (Chisti 2007), but the biomass growth was not limited by culture pH. With higher urine dilutions (1:150–1:300, **Figure 5.7B**) and with urine+TE (**Figure 5.7A**) pH remained lower during the whole incubation time, apparently due to ammonium uptake by algae (Brewer and Goldman 1976). Low pH values (<4) have resulted in cessation of *C. vulgaris* grown with ammonium (Hulatt *et al.* 2012), while the growth limiting pH for *C. vulgaris* is 3.5–4.5 (Huss *et al.* 1999); this supports the deduction of cultures not being affected by pH. Urea uptake by algae should not affect the culture pH (Brewer and Goldman 1976). Some urinary tract bacteria produce ureolytic enzymes (Krajewska 2009) which during the storage of urine increase the pH up to 9, leading to urea transforming into ammonium. The ureolytic activity of bacteria has likely caused the release of ammonium, which may also have increased the cultivation pHs.

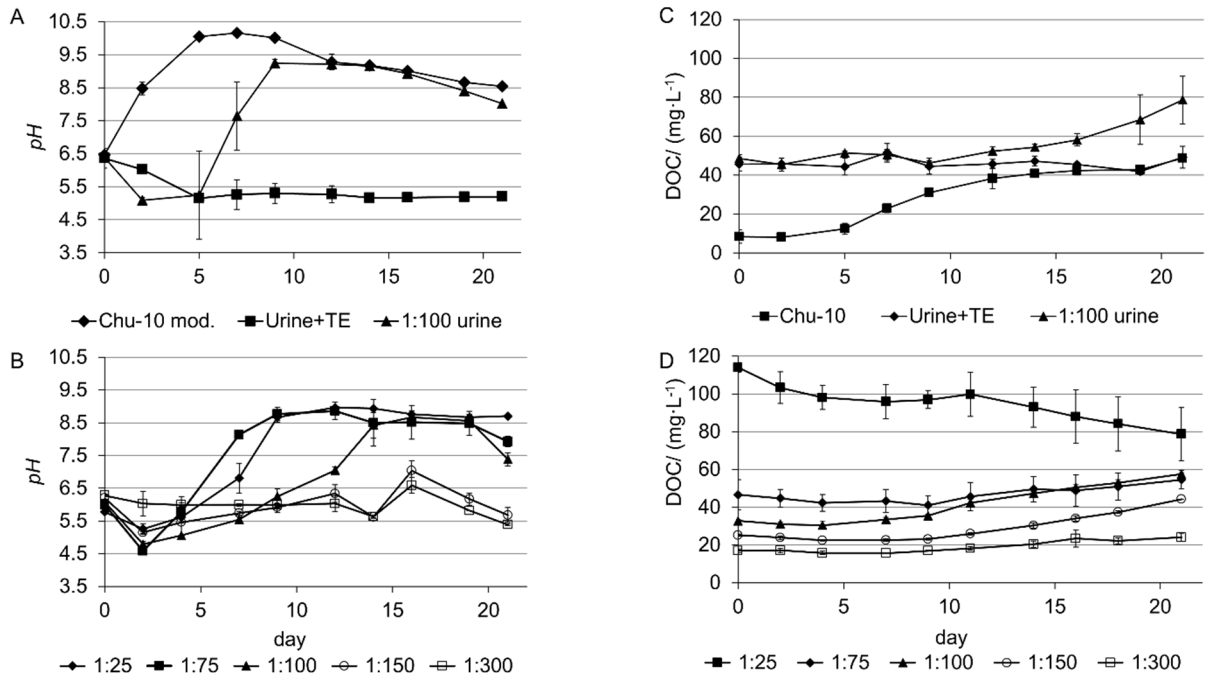

FIGURE 5.7. Cultivation pH: A) set-up I with 1:100-diluted urine, urine+TE and Chu-10 medium, B) set-up II with different urine dilutions. Dissolved organic carbon (DOC): in set-up I (C) and set-up II (D) at different urine dilutions. The error bars represent the standard error of three (A, C) and two (B, D) replicates. (Modified from **Paper IV**)

The DOC (initial concentration 8.4–114 mg/L) originated from the inoculum (**Figures 5.7C** and **5.7D**). In 1:25-diluted urine DOC decreased throughout the experiment, while the DOC in the other four dilutions began to rise after ten days (**Figure 5.7D**), which was expected, as all healthy algae release DOC during photoautotrophic growth (Puddu *et al.* 2003, Hulatt and Thomas 2010) and can also act as a substrate to heterotrophic microbes (Puddu *et al.* 2003 and references therein, Hulatt and Thomas 2010). DOC normally rises during *C. vulgaris* growing process, which has been explained by the production of extracellular polymeric substances that cannot be degraded by *C. vulgaris* (Babel *et al.* 2002). In heterotrophic growth, the algae use organic carbon for their growth, resulting in declining DOC concentration (Miao and Wu 2004). In 1:25-diluted urine, the concentration of DOC originated from urine was so high that the DOC released by *C. vulgaris* could not be detected. High reduction of DOC in 1:25-dilution was probably due to bacterial metabolism as it has been demonstrated that decrease of DOC in unsterilized wastewaters in *C. vulgaris* cultivation was due to bacterial activity (He *et al.* 2013a).

Effects of Nutrients on Biomass Growth

Nutrient utilization of the biomass was 33–57% of total nitrogen (N_{tot}) and 94–99% of P_{tot} in set-up I and 40–79% of N_{tot} and 35–92% of P_{tot} in set-up II, respectively. Decreasing P_{tot} concentration in the cultivations followed the algal growth (**Figures 5.8A** and **5.8B**) in both set-ups slowing simultaneously, suggesting that the algae might have utilized phosphorus in their growth, while the dead algae might have released phosphorus for the bacteria to utilize.

The N_{tot}, as well as organic nitrogen (N_{org}) concentration continued to decrease throughout the cultivation with diluted urine with increasing ammonium concentrations, while in cultivation where no urine was amended, no such obvious trend was seen (**Figures 5.9A–F**). *C. vulgaris* most probably utilized urea as it lacks ureolytic enzymes (Hodson and Thompson 1969) and metabolizes urea by urea carboxylase (Perez-Garcia *et al.* 2011b), while ammonium nitrogen (NH_4-N) formation was likely of bacterial origin. Since VSS_{algae} reached a stationary phase after around ten days, it is reasonable to assume that part of the urea consumption after that was due to bacterial metabolism. The source of nitrogen (urea or nitrate) has been shown to have little effect on the protein and lipid content or maximum biomass production of *C. vulgaris* (Hulatt *et al.* 2012, **Table 5.8**). Comparison of cultivation of different microalgae in urine and their biomass yields and growth rates (**Table 5.8**) demonstrated that the obtained biomass yields in urine cultivation were comparable with previous studies.

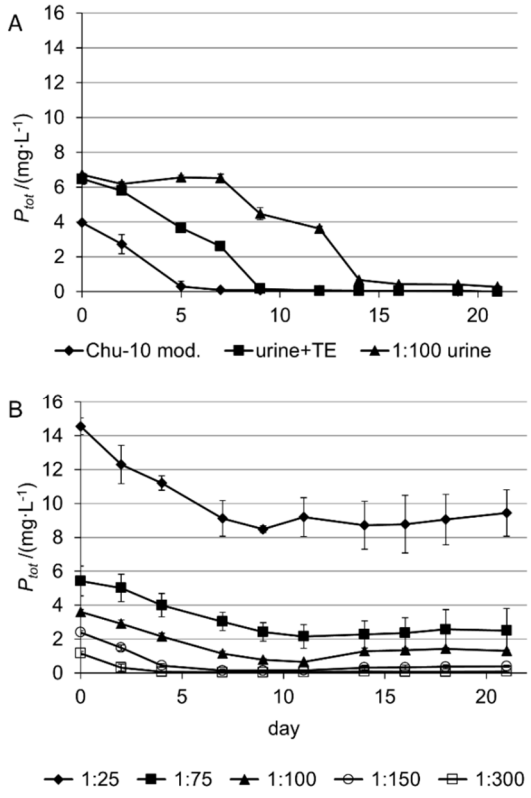

FIGURE 5.8. Phosphorus utilization (P_{tot}). A) set-up I with 1:100-diluted urine, urine+TE and Chu-10 medium, B) set-up II with different urine dilutions. The error bars represent the standard error of three (A) and two (B) replicates. (**Paper IV**)

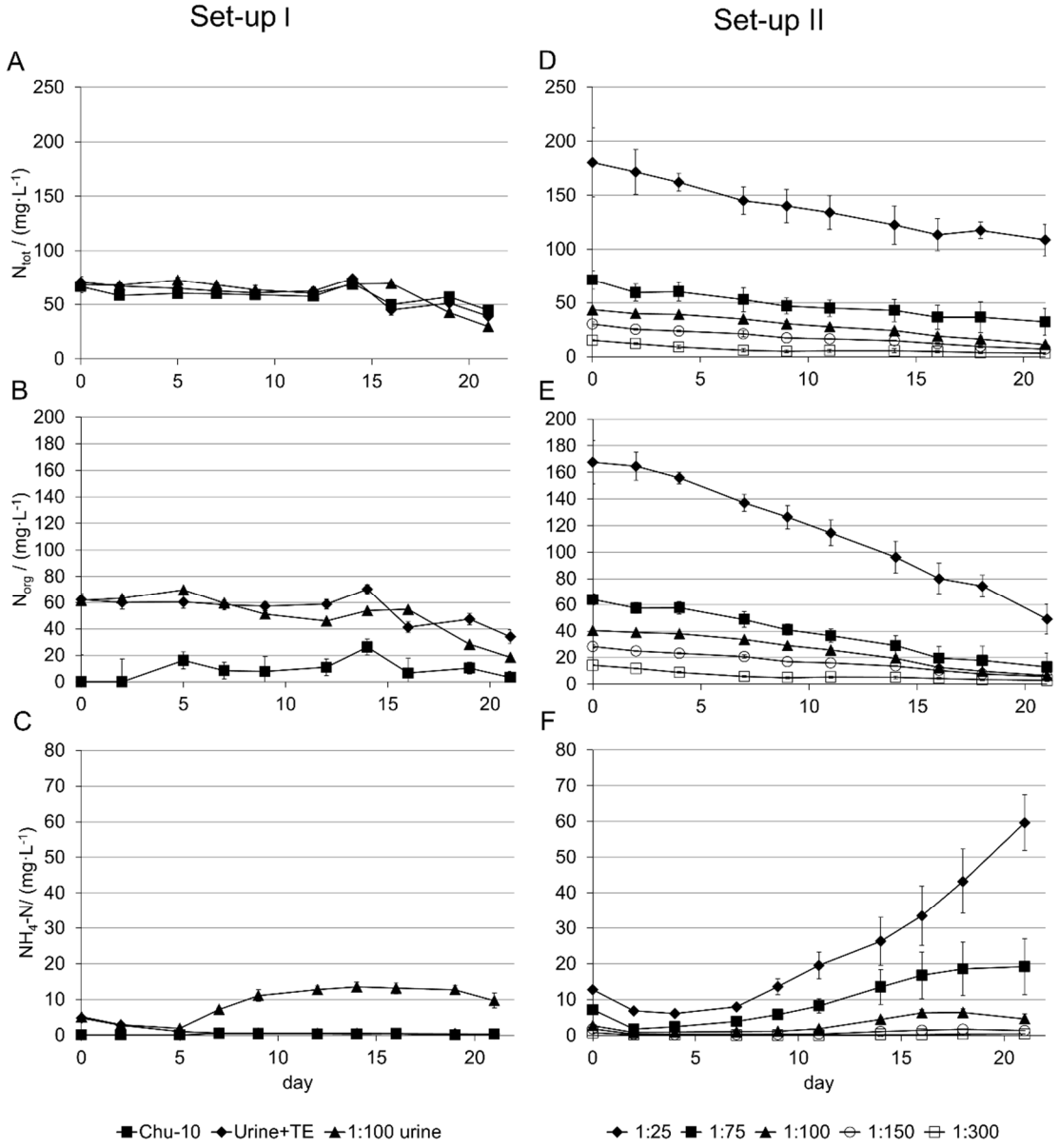

FIGURE 5.9. Nitrogen utilization as N_{tot}, N_{org}, and NH_4-N in set-up I (A, B, and C) and with different dilutions in set-up II (D, E, and F). The error bars represent the standard error of three (set-up I) and two (set-up II) replicates. (**Paper IV**)

The carbon to nitrogen (C:N) ratios in the cultivations ranged from 0.15 to 0.83 in cultivations with urine and artificial growth medium and from 0.74 to 1.3 in cultivations using diluted urine. The higher the C:N ratio is, the higher lipid production can be achieved in heterotrophic microorganisms (Silaban *et al.* 2014). Urea limitation (< 0.100 g/L) has been shown to increase *Chlorella* sp. lipid production while affecting negatively on the biomass concentration (Hsieh and Wu 2009). If the aim would be to use the produced biomass in biofuel production, optimization of the lipid content should be considered further, thus requiring a balance between sufficient nutrients while limiting the nitrogen concentration.

Especially the effects of urea from urine and thus ammonium as nitrogen source on the growth of algal cultures were of interest as previously it has been shown that ammonium uptake lowers the culture pH, thus affecting algal growth (Hulatt *et al.* 2012). Furthermore, unionized ammonia (as NH_3) can be toxic to microalgae at concentrations over 28 mg/L at pH over 8 when ammonium transforms into ammonia (Azov and Goldman 1982), as shown by Adamsson (2000) who reported decreased microalgal growth as ammonia concentration increased (2% urine was used). Contrary to this, *C. vulgaris* has been shown to prefer ammonium over nitrate and urea as a nitrogen source (Schuler *et al.* 1952, Perez-Garcia *et al.* 2011b). It was discovered that although ammonium was formed during cultivation in human urine (up to >59 mg/L in set-up II), it did not affect the overall biomass (incl. bacteria) production. *C. vulgaris* has been reported to have grown in ammonium concentrations up to 207 mg/L, and the higher ammonium concentration resulted in increase in protein content but when applied in excess, carbohydrate productivity was reduced (He *et al.* 2013b).

5.5. The Big Picture Based on the Results Presented in This Thesis

Based on the findings presented in this thesis, it is convenient to speculate about the issues in a bigger picture. The following discussion highlights the selected aspects of the source-separation of urine, analytical techniques on its characterization, pharmaceutical behavior, and utilization of urine. However, it is important to notice that the systems related to urine separation and use are complex, and they need more sophisticated approaches in order to assess e.g. the sustainability and safety of comparative systems. Currently, in the industrialized countries the sustainability of the sanitation practices needs to be assessed as the sewer infrastructure is on continuous development both in existing and new (residential) areas. As regards the less developed countries, necessity drives different sanitation customs and the threshold to employ sustainable and safe practices in order to protect people's livelihood and the environment is a pressing matter.

Pilot projects on urine collection and nutrient utilization are in operation in different parts of the world, as discussed in **Chapter 2**, and steps have been taken to make nutrient recovery from urine cost-effective while taking care of the hygienic quality. Yet, much needs to be done to improve the sanitation situation to the level where hygienic aspects are tackled with and focus can be directed to ecological aspects - therefore, in order to illustrate urine as a resource, an example scenario on urine collection and resource utilization in a Nordic climate is presented in **Example 1**.

Example 1: Collection and Storage of Urine in a Larger Scale

In a community of 10 000 people, and urine production of 1.2 L/person/d, 12 m^3/d urine is produced. On an annual level, this means 4380 m^3 of urine. In a community, the joint collection of urine e.g. from adjacent or closely located buildings could be applied. From separate collection vessels, urine could be e.g.

transported to a larger collection facility. For example, three or four 1000 m^3 tanks would be enough for six-month urine storage in appropriate environments before fertilizer application, and the tanks could be filled such that the order of filling rotates – as one tank is filled e.g. during three months, the second tank will start to fill after that and the tanks can be rotated in use. In this manner, the recommended six-month storage is applied on collected urine with minimum storage. For example, barley, wheat, and oats at Nordic climate require on average 100 kgN/ha and 38 kgP/ha (meta-analysis by Valkama *et al.* 2013). When applying the average nitrogen concentration in collected urine (**Table 5.1,** 5.56 gN/L), the amount of N in 12 m^3/d urine would be equal to 67 kgN/d. During six months, the amount of collected urine would contain approximately 12 tons N which would be enough to fertilize 120 ha field area equaling 240 ha field area per year. In the case of phosphorus, 12 m^3/d urine would contain 6.2 kgP/d (0.513 gP/L, **Table 5.1**) making it a little over 1 ton of P which could be used to fertilize 29 ha field equaling 59 ha annually. Therefore, the amount of phosphorus in urine is limiting nutrient.

The findings in urine nutrient concentrations (**Papers II–III)** suggest that while urine is a potential renewable fertilizer source, the pharmaceutical compounds and estrogenic activity present in urine after storage are an aspect that still needs to be addressed using e.g. enhanced treatment methods in order to promote both nutrient recovery and harmful compound removal. When considering controlling the use of urine in fields, the monitoring tools used in **Papers I** and **II** could come handy in faster analysis and preliminary screening for legislative bodies, research facilities and on-site inspectors. The HPLC method and bioluminescent yeast biosensor used for urine characterization in this thesis (**Papers I** and **II**) are quite robust. The HPLC method can be applied in places were access to more accurate systems (e.g. HPLC-MS/MS) is not available, as the method operates in the range of real pharmaceutical concentrations in urine. For lower concentration range and transformation product identification other tools are required. As for the yeast biosensor, the portability is a prospective that could be true in the near future. Main advantage of these techniques would probably be for the monitoring personnel that control the fertilizer quality.

The presence of feces during urine storage (**Paper III**) affected negatively on pharmaceutical removal as did the urease inhibitor. Feces are practically always present as contamination and it is probably quite difficult to prevent such contamination in practical use of separating toilets. The results presented in this thesis apply, however, only to the selected compounds and it is possible that in the case of other pharmaceuticals the concentration reductions could be higher.

As for the urease inhibitor (**Paper III**), since it was still 59% present in urine after six months, it is quite possible that the bacterial activity of the urine was disturbed thus leading to the low concentration reduction results. The disruption of bacterial activity, on the contrary, could have affected the high remaining concentration of the urease inhibitor after the storage period. At a

practical point of view, if urine containing such a high concentration of urease inhibitor would be applied in agricultural fields, it might cause the disruption of bacterial enzymes in the soil, as was speculated by Watson *et al.* (2008). Therefore, before more is known about the urease inhibitor effect on soil activity, its use should probably be limited to urine which doesn't contain pharmaceuticals, in order to achieve reduction in ammonia volatilization in the urine in the soil, as they are planned to do. In the scenario presented in **Example 1**, the use of urease inhibitors in preventing collection pipeline blockages due to struvite could be applied (see Tampere University of Technology *et al.* 2011).

Microalgal systems are commercially available for bioactive compounds but not yet in a larger scale e.g. for high rate algal ponds in wastewater treatment (Jegathese and Farid 2014). The microalgal cultivation systems come in different shapes and sizes, but the scaling up is still in its infancy and more research is needed to make larger microalgal cultivations profitable. Reactor experiments with urine have shown promising results, but with the aid of multiple additives, the cost of which need to be considered.

The nutrient and water sources can have a large impact on microalgal cultivations costs, as well as the selection of microalgal species, climate conditions, existing infrastructure, and logistic consideration (Rawat *et al.* 2013) as well as biomass harvesting and additional illumination (Cho *et al.* 2013). To give some perspective of the algal and related biomass production in a larger scale, the following example (**Example 2**) was constructed.

Example 2: Biomass Production Potential of Microalgae

To estimate the produced biomass in a residential area of 10 000 inhabitants, each producing 1.2 L of urine per day was assumed. The results obtained in **Paper IV** and urine composition from set-up II (**Table 5.1**) were used in the calculations. The available N_{tot} and P_{tot} in liquid form for biomass growth would be 23.5 tons/a and 2.2 tons/a equaling 170 tons/a and 230 tons/a of biomass, respectively. This biomass would provide suitable feed e.g. for anaerobic digestion. 1 tonVS provides 200-400 m^3 of methane (CH_4) (in anaerobic digestion for different algal biomass sources, Gunaseelan 1997) and for *C. vulgaris* 286-322 m^3 CH_4/tonVS (Lakaniemi *et al.* 2011, Lü *et al.* 2013), thus corresponding to approximately 170 tonsVS/a × 300 m^3 CH_4/tonVS = 50939 m^3 CH_4/a, which could be used e.g. in biofuel or biogas production, equaling 0.51 GWh. If lipids are extracted before anaerobic digestion, the potential methane yield is lower while the released ammonium is higher (Sialve *et al.* 2009). Yet, it must be kept in mind that biogas production itself also consumes energy while the digestate can be considered in fertilizer use.

The water potentially needed for the dilution of urine for the algal cultivations needs to be considered as use, for instance, of drinking water seems questionable and more sustainable sources for water should be considered. The algae and other microorganisms would remove most of the nutrients in the cultivation medium, but it is possible some post-treatment for the

water would be required before release to the environment. As previously presented in this thesis, the possible flocculation of algae with bacteria, and overall bacterial contamination would probably render the dilution water useless to be reused as substances remaining from bacteria might cause algal clumping at the beginning of the next cultivation. A frequent cleaning of the cultivation vessel would be required to ensure the optimum growth of microalgae (Lam and Lee 2012b) due to bacterial presence.

6 Conclusions and Recommendations for Future Research

Based on the results and findings presented in this thesis, the following conclusions and research recommendations can be constructed:

- Properties of source-separated urine are favorable for fertilization purposes due to its relatively high nutrient concentrations (N, P, K) and renewability (**Papers II**, **III**, **and IV**).

- Analysis of urine characteristics and pharmaceutical and estrogenic monitoring can be enhanced due to development of a HPLC method for simultaneous pharmaceutical detection (**Paper I**) and applicability of the yeast biosensor for urine analysis without the need for pre-treatment or adaptations of the biosensor for urine matrix (**Paper II**).

- HPLC detection provides pharmaceutical detection range feasible for urine analysis of the selected pharmaceuticals (**Paper I**), while the yeast biosensor assay provides reasonable information on estrogenic activity in order to state the time when the activity has been reduced adequately in order to re-use urine (**Paper II**) and prevent possible migration into the soil or water bodies.

- The yeast biosensor produces a cumulative signal which includes both natural and synthetic estrogens and estrogen-like compounds in urine (**Paper II**), therefore enabling exposure assessment of anthropogenic EDCs in urine.

- Urine storage period of six months recommended by WHO is not enough to reduce the studied pharmaceutical concentrations (10–80 mg/L, three antivirals and four antibiotics) (**Paper III**).

- Chemical or biological removal of antibiotic/anti-tuberculotic compounds can be expected to some extent in source-separated urine (**Paper III**), but the behavior of antivirals needs more investigation. In addition, antiviral resistance of viruses in the

environment has risen as one of the major concerns regarding pharmaceutical emissions from WWTPs. Methods for decreasing pharmaceutical concentrations in stored, source-separated urine should be studied further to enable its safe use. Additionally, the mechanisms affecting the removal require more research.

- A potential for transformation product formation is evident (**Paper III**). Findings support the fact that using (stored) human urine directly in agricultural fertilization for edible plants may pose a risk. Thus, other feasible treatment practices or urine utilization methods should be considered.

- Pharmaceutical concentrations are not reduced markedly during urine storage (**Paper III**). The treatment of the liquid phase remains a challenge for technical realization. However, separating urine from the municipal wastewater stream would no doubt aid in improving the effluent quality and the quality of the receiving waters, naturally depending on the place and/or use of urine being conveyed.

- Presence of urease inhibitor, commonly used in fertilization applications to inhibit ammonia volatilization, affected negatively on pharmaceutical removal (**Paper III**). The effect of urease inhibitor on bacterial enzyme activity during urine storage needs thus more consideration. In addition, fecal contamination has negative impact on pharmaceutical removal, which can prove problematic in real-life applications.

- Microalgae can grow in 1:25–1:300 times diluted human urine (**Paper IV**). The diluted urine is able to support biomass growth without additional trace elements or vitamins, thus having potential for sustainable use and recycling of nutrients.

- Microorganisms present in the collected urine (**Paper IV**) can facilitate algal cell flocculation when re-using the urine-based cultivation medium. However, cultivation of *C. vulgaris* in more concentrated urine and the effect of the selected pharmaceuticals and estrogens on its growth need more research, as no studies on pharmaceutical-containing urine on algal growth have been conducted.

- The water coming for dilution of the algal cultivations is one major aspect. Microalgal (and related biomass) cultivation using only little diluted, source-separated urine could in the future be a sustainable way to re-use nutrients and generate biomass for energy production (**Paper IV**).

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ORIGINAL PAPERS

I

SIMULTANEOUS DETECTION OF THREE ANTIVIRAL AND FOUR ANTIBIOTIC COMPOUNDS FROM SOURCE-SEPARATED URINE WITH LIQUID CHROMATOGRAPHY

by

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Sanna T. Pynnönen[1]
Tuula A. Tuhkanen[1,2]

[1]Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland
[2]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

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Research Article

Simultaneous detection of three antiviral and four antibiotic compounds in source-separated urine with liquid chromatography

An analytical method for the simultaneous screening of three antiviral agents (nevirapine, zidovudine, lamivudine), four antibiotics (sulfamethoxazole, trimethoprim, ciprofloxacin, rifampicin) and one reference compound (carbamazepine) in human urine was developed. Separation was achieved with a Kinetex XB-C_{18} (75 × 4.6 mm, 2.6 μm) column after the extraction of pharmaceuticals from urine with SPE. Gradient elution with a mobile phase consisting of acetonitrile and 10 mM KH_2PO_4 (pH 2.5), and diode array detection with monitoring at 210 and 264 nm was applied. The developed method was validated in terms of selectivity, linearity, stability and sensitivity. Repeatability ($n = 3$) and between-day precision ($n = 3$) revealed RSD <5%. The detection limits were estimated as 0.02–0.54 g/L (depending on compound). The method was validated for human urine and successfully applied to the simultaneous quantification of selected compounds. Strata-X cartridges provided good recoveries ranging from 81 to 109%. The limits of detection for urine varied between 0.04 and 1.61 g/L. The method is suitable for the fast determination of selected pharmaceuticals from source-separated urine samples for further environmental risk assessment and degradation potential evaluation. It provides a way to enhance safe nutrient recycling from wastewater streams and promotes the safe use of urine as fertiliser.

Keywords: High-performance liquid chromatography / Pharmaceuticals / Solid-phase extraction / Sustainable sanitation / Urine
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1 Introduction

In many countries, mixing all wastewater streams together before treatment is common. Urine makes up only ∼1% of the amount of wastewater arriving at wastewater treatment plants but comprises the main portion of all the nutrients (80% N, 50% P, 90% K) [1–3]. Urine also contains the residues of the consumed pharmaceuticals, either as a parent compound or as metabolites. Pharmaceuticals may not be removed during wastewater treatment or metabolites can be transformed back into active compounds by bacterial metabolism. A way to (i) enhance the nutrient recycling and (ii) remove the pharmaceuticals simultaneously is to collect the urine fraction separately (source separation).

The pressure for resource recovery has made the fertiliser use of source-separated urine an attractive alternative to chemical fertilisers. Yet, the precautionary principle has prevented the widespread use of urine as fertiliser, since the fate of pharmaceuticals present in urine, when released to the environment, is unclear. This problem overlaps with the high prevalence of demand for low-cost nutrients, which is a significant issue particularly in developing countries.

The environmental and health risks caused by pharmaceutical residues in urine are currently neglected. However, from the perspective for future fertiliser use [4], it is important to monitor their presence in the potential nutrient source. Therefore, if urine is used as a fertiliser it should be ensured that the pharmaceuticals in human urine do not pose a threat to humans or to the environment. Currently, the main focus in related studies has been in either biological fluids for bioanalytical purposes [5, 6] or in environmental analyses where pharmaceuticals are commonly dealt in groups of similar analyte properties or each compound is detected with a different chromatographic method [7]. Also,

Correspondence: Sanna T. Pynnönen, Department of Chemistry and Bioengineering, Tampere University of Technology, PO Box 541, 33101 Tampere, Finland
E-mail: sanna.pynnonen@tut.fi
Fax: +358-3-364-1392

Abbreviations: **3TC**, lamivudine; **CBZ**, carbamazepine; **CIP**, ciprofloxacin; **DAD**, diode array detector; **NVP**, nevirapine; **RMP**, rifampicin; **SMX**, sulfamethoxazole; **TRI**, trimethoprim; **ZDV**, zidovudine

attention has been directed to compounds commonly associated with the high standards of living, such as lipid-modifying agents, analgesics and anti-rheumatics, and beta blockers [8], but pharmaceuticals relating to HIV treatment have received less consideration.

In developing countries, antiviral drugs to treat HIV and antibiotics to treat tuberculosis are widely used. Three antiviral drugs nevirapine (NVP), zidovudine (ZDV) and lamivudine (3TC) are commonly used as fixed-dose combinations to treat HIV [9]. Antibiotics sulfamethoxazole (SMX) and trimethoprim (TRI) are used alongside antivirals to prevent HIV-induced infections: approximately 76% of HIV patients in sub-Saharan Africa use this combination called 'cotrimoxazole' (http://www.aidsmap.com/TB-still-killing-4000-people-with-HIV-each-day-WHO-reports/page/1543089/, [10]). Antituberculotic pharmaceuticals rifampicin (RMP) and ciprofloxacin (CIP) are commonly used to treat tuberculosis patients. Many HIV patients have latent tuberculosis infection that is activated by HIV and lowered immunity. Carbamazepine (CBZ) was selected in this study as a recalcitrant reference compound. All pharmaceuticals are excreted in urine at different percentages of the ingested oral dose, which makes it interesting to monitor their concentrations in urine. Since source-separated urine should be stored for at least six months before use to remove pathogens [11], the monitoring of pharmaceuticals is especially interesting if urine is meant for fertilisation purposes.

These selected compounds have been detected in both wastewater [12, 13] and surface water [14]. The detection of some of these has been achieved using HPLC–MS [12, 14–16] and GC–MS [17]. To the best of our knowledge, there is little information available regarding RMP detection from wastewaters. Antiviral compounds have been recognised as recalcitrant and persistent in the environment (for a review, see ref. [18]). Antiviral compounds have been detected from wastewaters using HPLC–MS [13]. However, pharmaceuticals found in these waters are extensively diluted and the compounds usually appear in the μg/L to ng/L range and are thus referred to as micropollutants [19]. In environmental matrices, sample concentration is often necessary [20].

HPLC has been used to detect these compounds from human serum [21, 22] or plasma [23]. RMP has been detected from antituberculotic tablets [24] and from human plasma and blood spots [25]. Antiretroviral drugs have been detected from plasma [26]. Some studies have also concentrated on human urine [27–30]. In the case of human urine, most pharmaceuticals are excreted in the mg/L range and can be more easily analysed by using only HPLC with diode array detection. However, to the best of our knowledge, there is no method available that simultaneously detects all of the selected pharmaceuticals from urine.

The detection of pharmaceuticals in plasma, urine and medicinal products has been one of the basic analyses performed in the medical industry. However, these analyses have relied on methods that utilise porous C_{18} columns with particle sizes of 3–5 μm. The use of a core–shell column enables fast gradient separations with a performance similar to ultra-performance liquid chromatography systems in the 400 bar pressure limitation of conventional HPLC systems [31, 32]. According to Fekete and Fekete [33], the performance achieved with 2.6–2.7 μm core–shell particles is comparable with results obtained with fully porous 1.7 μm particles when utilising fast gradient separations. Their results showed that this technology enables fast and efficient gradient separations in routine pharmaceutical analysis.

Previous studies [34, 35] have demonstrated the good performance of 2.6 μm Kinetex-C_{18} particles under gradient conditions. The column selected for this study utilises a XB-C_{18} phase (octadecyl silane) that, according to the manufacturer, increases hydrogen bonding with hydrophobic selectivity, which improves the peak shape for basic compounds and increases the retention of acidic compounds.

Our preliminary studies showed that the chromatogram of a urine sample contains several distinctive peaks created by the urine matrix and therefore the sensitivity of the method is not sufficient at low concentrations. SPE provides a rapid and easy means of extraction and purification of urine samples. A previous study by Lai et al. [36] proved that urine samples treated with SPE had less interfering peaks that could hinder the identification of compounds of interest in the chromatogram. Therefore, the analysis of urine samples with an HPLC instrument without any sample pre-treatment is not an option since the detector cannot distinguish individual compounds from the interfering peaks caused by the urine matrix.

The aim of this study was to develop a simple and rapid HPLC gradient method utilising a core–shell column to screen all the selected pharmaceuticals from human urine simultaneously in the 0.05–500 mg/L range. This range is relevant to the calculated predicted concentrations in urine. The method enables the fast determination of selected pharmaceuticals from collected urine samples for further environmental risk assessment and degradation potential evaluation.

2 Materials and methods

2.1 Chemicals and materials

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma-Aldrich (Sweden). Potassium dihydrogen phosphate, ammonium hydroxide and acetic acid were of analytical grade and purchased from Merck. Phosphoric acid was purchased from VWR (Prolabo). NVP, ZDV, 3TC, RMP, CIP, SMX, CBZ and TRI were received from United Corporation (Kenya). MilliQ-water was de-ionised water with 18.2 mΩ cm conductivity (Millipore). The physicochemical properties and type of usage of the analytes are presented in Table 1. They range from acidic to neutral, basic and zwitterionic compounds. The chemical structures of the compounds are presented in Fig. 1. Stock solutions of 1.0 g/L were prepared in MeOH, except CIP in MilliQ-water because of low solubility in MeOH. CBZ stock solution concentration was 0.1 g/L. The stock solutions were stored in dark glass

Table 1. Properties of the selected pharmaceuticals

Compound		CAS	Chemical formula[a)]	M[a)] (g/mol)	pK_a[a),b)]	logK_{ow}[a),b)]	Water solubility[a),b)] (g/L)	Typical usage
Lamivudine	3TC	134678-17-4	$C_8H_{11}N_3O_3S$	224.26	4.3	−1.29	2.76	Antiviral (HIV-treatment)
Zidovudine	ZDV	30516-87-1	$C_{10}H_{13}N_5O_4$	267.24	9.96	0.05	16.3	Antiviral (HIV-treatment)
Nevirapine	NVP	129618-40-2	$C_{15}H_{14}N_4O$	266.89	2.8	1.75	0.105	Antiviral (HIV-treatment)
Sulfamethoxazole	SMX	723-46-6	$C_{10}H_{11}N_3O_3S$	253.28	1.6; 5.7	0.89	0.459	Antibiotic
Trimethoprim	TRI	738-70-5	$C_{14}H_{18}N_4O_3$	290.32	7.12	0.91	0.400	Antibiotic
Ciprofloxacin	CIP	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.35	6.09; 8.74	0.28	30	Antibiotic (tuberculosis)
Rifampicin	RMP	13292-46-1	$C_{43}H_{58}N_4O_{12}$	822.94	1.7; 7.9	4.24	1.4	Antibiotic (tuberculosis)
Carbamazepine	CBZ	298-46-4	$C_{15}H_{12}N_2O$	236.27	13.9	2.45	0.0177	Antiepileptic

Data collected from
a) DrugBank (http://www.drugbank.ca/).
b) Toxnet (http://toxnet.nlm.nih.gov/).

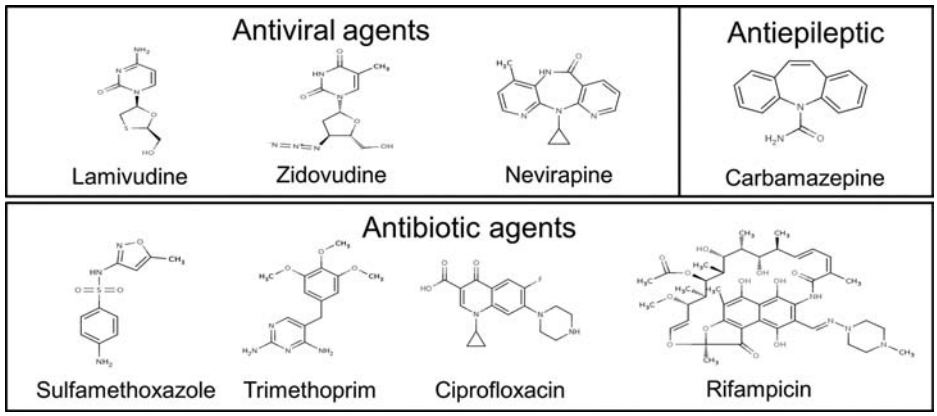

Figure 1. Chemical structures of the studied compounds. Pictures obtained from DrugBank (http://www.drugbank.ca/).

containers at about 4°C, and they were usable for several months. Urine samples were collected from healthy volunteers receiving no medication. The samples were combined and carefully mixed, afterwards the samples were spiked. Analysis was performed on the same day as samples were collected.

2.2 Liquid chromatography

Analysis was performed using Hewlett-Packard Agilent 1100 HPLC (Agilent Technologies) consisting of a binary pump, a vacuum degasser, an autosampler, a thermostatted column oven kept at 40°C, a standard flow cell (13 μL) and diode array detector (DAD). The temperature of the column oven was set at 40°C to decrease the effect of room temperature variation on the column performance and to achieve a lower operating pressure due to the decreased viscosity of the mobile phase. All capillaries were of 0.17 mm internal diameter. The dead volume of the system was 24.4 μL. The column used to perform analyses was Kinetex core–shell XB-C_{18}, 2.6 μm (100 Å), 75 × 4.6 mm (Phenomenex, Denmark) equipped with SecurityGuard ULTRA Pre-filter cartridge 0.2 μm (Phenomenex). Data analysis was conducted using HP ChemStation software version B.01.03.

The mobile phase was selected based on preliminary studies conducted in our department earlier (data not shown here). The mobile phase composition had been shown to be effective in the detection of pharmaceutical compounds from wastewater samples. The mobile phase consisted of two parts: eluent A was ACN and eluent B 10 mM KH_2PO_4 buffer filtered through a 0.2 μm filter (ME24, Schleicher & Schuell, Germany), the pH of which was adjusted to 2.50 with 85% H_3PO_4. The peaks were detected using a DAD at wavelengths 210 nm (3TC, TRI, ZDV, CIP, NVP, SMX, CBZ) and 264 nm (RMP), and peak purity was determined by running DAD from 190 to 600 nm during each analysis to specify the spectra for each compound. The data acquisition rate was set to the highest possible (<0.1 s). The injection volume was 5 μL, and the flow rate of the mobile phase was 2.5 mL/min. The high flow rate was selected with the core–shell particle column to provide optimum performance and sharp peaks with the Agilent 1100 system, but also to achieve the separation of the compounds in a short analysis time. Column backpressure during the sample run was approximately 330 bar.

The method development was started with a linear gradient of 5–90% eluent A in the selected time and modified repeatedly to obtain the best selectivity between different pharmaceuticals, resulting in the gradient presented here. Before

running the samples, the UV signal at 210 nm was left to stabilise with 5% eluent A flow. Gradient elution was used: isocratic hold from 0.0 to 0.5 min at 5% eluent A, linear gradient from 0.5 to 3.5 min from 5 to 70% eluent A, after which an isocratic hold at 70% eluent A from 3.5 to 4.0 min was applied. The gradient elution took place in 4.0 min and the signal was left to stabilise for 2.0 min at 5% eluent A after each sample. The total analysis time was 6.0 min.

2.3 Quantification

Each compound was identified by recording the retention time and spectrum of the pure compound in MeOH and comparing those to each peak in the sample. The calibration curves were made in the range of 0.05–500 mg/L for each compound from stock solutions straight into amber vials by serial dilution. Internal standards were not used, since the large variability of the therapeutic groups in one sample would cause difficulty in internal standards selection and possibly hinder the analysis of multiple compounds from one sample.

Standard curves were prepared by plotting peak areas of the target analytes versus analyte concentrations. A linear regression model utilising the least-squares fitting (Microsoft Excel 2007) was applied to the results and the calibration curves were stored for further use of the method.

2.4 Application to urine matrix

Urine samples were spiked with a known amount of pharmaceutical, vortexed and particles in the urine samples were removed by filtering the spiked sample through a 0.2 μm nylon syringe filter (VWR, USA) to protect the column. The first few drops of filtrate were discarded. Afterwards 1 mL of sample was pre-treated by SPE.

2.5 SPE

Based on preliminary studies (data not presented here), a choice from three different SPE sorbent types was made on the basis of how well they removed interferences caused by the urine matrix. Selected pharmaceuticals were extracted from urine samples using Strata-X (1 cc/30 mg, 33 μm) SPE cartridges from Phenomenex, which enabled the simultaneous determination of selected compounds. Strata-X sorbent consists of chemically modified styrene-divinylbenzene (mixed polymeric and reversed phase [37, 38]). It has not been as extensively used as some other sorbents, such as Oasis HLB (Waters), but Strata-X has performed well, for example, in bovine [39] and human urine pre-treatment [37].

SPE was conducted using a vacuum manifold (Supelco Analytical, USA). The method was based on manufacturer's instructions for Strata-X (http://phx.phenomenex.com/lib/tn75040410_l.pdf) in which the wash and elution steps were modified. Extraction volume for urine was 1 mL. SPE sorbent tubes were conditioned with 1 mL MeOH, equilibrated with 1 mL MilliQ-water, after which 1 mL of sample was introduced. After sample loading, the sorbent was dried under vacuum for 1 min, eluted with 1 mL 2% NH_4OH to collect 3TC and dried under vacuum for 1 min. The second elution was conducted with 1 mL of ACN/MeOH/acetic acid (50:50:2, v/v/v) to collect the rest of the compounds. The flow rate was approximately 1 mL/min. The eluted samples were collected into amber vials for analysis.

2.6 Method validation

To determine the instrumental quantification limit, the LOQ and LOD were determined from standard solutions. In this study, we used $S/N = 3$ for the LOD and $S/N = 10$ for the LOQ. The LOD and LOQ were also determined for each compound separately in the urine matrix.

The robustness of the method was tested by running two different gradient slopes compared with the method. The gradient was modified by adjusting the eluent A end-concentration either to 60 or 85% and the changes in compound retention times (t_R) and peak areas were recorded. In addition, the effect of eluent A starting percentage was studied. The effect of temperature was monitored by testing sample run temperatures ± 5°C, and the same data was collected as with gradient change. The effect of pH on selectivity was also tested at pH values ± 0.5 units past the optimum (pH refers to the pH of the aqueous buffer).

The reproducibility of the method was verified by statistically comparing the concentration found in the sample with the one added. Statistical analysis was performed by conducting a two-tailed one-sample *t*-test (SPSS 15.0, IBM) using the added concentration as test value and studying the *p* value of the results. The null hypothesis (H_0) was that there is no difference between the added and found concentrations. If a *p* value of >0.05 is observed, it verifies that the difference in concentrations is not statistically significant.

The repeatability of the LC method was tested by triplicate injections of standard concentrations (1.0, 10.0 and 100.0 mg/L in MeOH and CIP in water) and spiked urine samples (the pharmaceutical concentrations of 5.0, 10.0, 25.0 and 50.0 mg/L). Spiking was done to a urine sample that did not contain pharmaceuticals with a mixture of selected compounds; the sample was filtrated through a 0.2 μm nylon filter and the SPE procedure was performed as previously described.

The recovery is the percentage of which known added concentrations of compounds were obtained from a matrix after pre-treatment and analysis. This was studied by using concentrations of 5.0, 10.0, 25.0 and 50.0 mg/L for selected compounds with three replicates each. The recoveries were determined by comparing the peak area of the SPE-treated sample to the peak area of the same compound at the same concentration level dissolved in MeOH or MilliQ-water. The LOD and LOQ were also determined for urine samples.

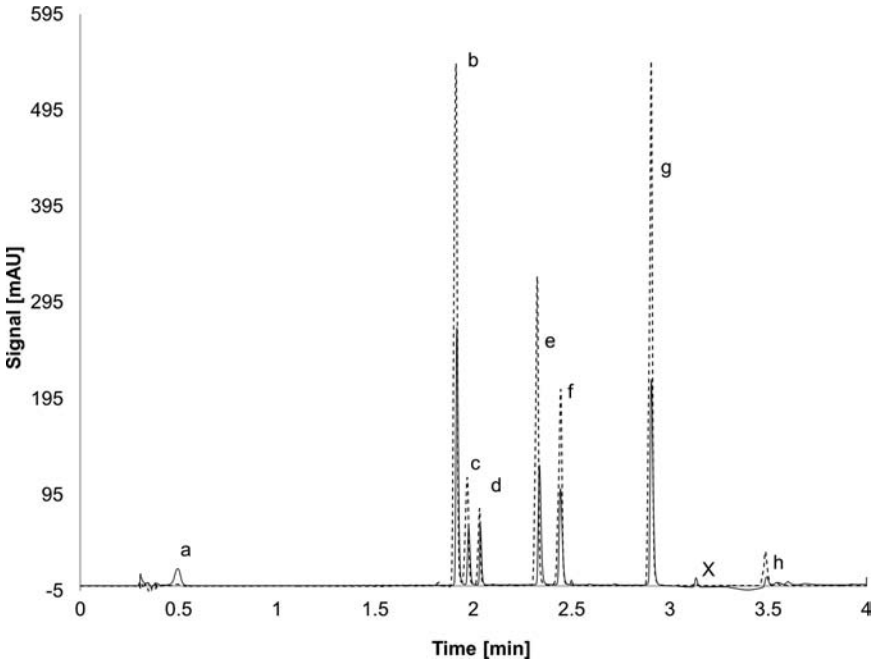

Figure 2. Chromatographic peaks of the selected compounds at 10.0 mg/L (standard solution, solid line) and SPE-treated spiked sample of 25 mg/L (dashed line, second elution with 50:50:2 ACN/MeOH/acetic acid). (a) lamivudine, (b) trimethoprim, (c) zidovudine, (d) ciprofloxacin, (e) nevirapine, (f) sulfamethoxazole, (g) carbamazepine, (h) rifampicin, (x) impurity caused by rifampicin. Chromatographic conditions: Eluent A acetonitrile, eluent B 10 mM KH_2PO_4, pH 2.5. Flow rate 2.5 mL/min, UV at 210 and 264 nm, injection volume 5 μL, column temperature 40°C, isocratic hold from 0–0.5 min at 5% A, gradient from 0.5–3.5 min 5–70% A, isocratic hold 3.5–4.0 min at 70% A.

3 Results and discussion

The developed method produced sharp peaks with acceptable resolution. Injection volumes of 3, 4, 5, 6, 7 and 10 μL were tested: < 5 μL injection volumes reduced the peak height too much and injection volumes > 5 μL caused less symmetric peaks and the resolution between different peaks suffered. Thus, a 5 μL injection volume was selected. In addition, the effect of the eluent flow rate was tested. At a flow rate of 2.0 mL/min the elution of the last peak (RMP) was delayed markedly. Gradient elution was set to take place in 0.5 min steps to decrease the analysis time. Subsequently, the flow rate was adjusted to 2.5 mL/min, which further reduced the analysis time to 4 min. DeStefano et al. [40] have demonstrated that the flow rate of the mobile phase can be substantially increased and the separation time decreased with only a small decrease in the column efficiency and separation resolution when utilising new sub-3 μm particle columns.

Figure 2 presents the order of the peak elution for the stock compounds and for the urine sample (sample taken from elution with 50:50:2 v/v/v, ACN/MeOH/acetic acid). Between peaks g and h there is a smaller peak (x), which is caused by RMP. Different gradient slopes and ACN end-concentrations were tested but they did not have an effect on the retention of CBZ and RMP. The retention times (t_R), the calibration curve R^2 values, α (selectivity), *k'* value (capacity factor), and LOD and LOQ of the instrument for each compound can be found in Supporting Information Table S1. Supporting Information Table S1 also describes the standard deviations of the calibration curves slope and intercept. The R^2 values were all ≥0.9992 and *k'* and α were acceptable. The standard deviations of the retention times were all < ± 0.02 min. The LOD and LOQ varied between 21–536 μg/L and 71–1786 μg/L, respectively, and depending on the compound.

The linear range of the calibration varied depending on the compound. The number of calibration points was seven for 3TC, eight for CBZ and CIP, nine for TRI, SMX and NVP, ten for RMP and 11 for ZDV. The capacity factor (*k'*) of a peak should preferably be <12, and in this case the capacity factor of 11.9 for RMP was accepted because of the highly reduced sample run time. Also, the *k'* value for 3TC was quite low (<2), but this could not be affected even by changing the gradient to start at 2% eluent A concentration. Any lower ACN percentage would even be harmful for the performance of the column. The selectivity (α) between peaks should preferably be at least 1.2–1.3, but in our analysis three peak pairs had selectivities of 1.0 (peak pairs b/c and c/d) or 1.1 (peaks e/f), see Supporting Information Table S1. However, they resolved well from each other. The reproducibility of the method was acceptable. The RSDs for intra- and interday tests (not shown here) were all <8%. A thinner particle shell means a smaller surface area of particles. This causes smaller retention values (*k'*) and reduced sample loading capacity [40], which might explain the behaviour of 3TC and the variability in the largest concentration level measurable at linear range, e.g. for TRI, concentrations over 100 mg/L, which resulted in the loss of linearity in the standard curve.

Supporting Information Table S2 presents the robustness results. In Supporting Information Table S2a, the effect of alternative temperature is presented. When alternating the column temperature, the changes in peak areas were within ± 10% and within ± 14% with temperatures higher and lower than 40°C, respectively. Supporting Information Table S2b describes the effect of gradient slope change. The gradient slope change did not affect the peak areas of the compounds markedly. The variation percentage was between ± 5%, which is acceptable. When using a higher percentage of eluent A (ACN), the peaks eluted approximately 0.1–0.4 min

faster than when using 70% ACN end-concentration. With a lower gradient, the t_R did not change, except for RMP, which eluted 0.4 min slower. However, the method was still capable of separating RMP within the 4.0 min time limit. Supporting Information Table S2c explains the effect of changing gradient start to 2% eluent A instead of 5%. The retention of the problematic compound 3TC was not improved with a lower starting ACN percentage. The changes in peak areas were negligible when using a different gradient start when compared with the gradient proposed in this article. The retention time shifts either faster (2–85% ACN) or slower (2–60% ACN) but the analysis could still be concluded within 4 min. The order of peak elution did not change in any of the cases. Supporting Information Table S2d describes the effect of eluent pH change to peak elution and selectivity. At pH 2.0, the t_R of the compounds were in the range of ± 0.05 min except for NVP (t_R was shortened by 0.12 min). At pH 2.0, the selectivity between peaks e and f (NVP and SMX) was increased, for all the other compounds it remained the same. The peak area changes were ± 7.2%. At pH 3.0, the retention times varied ± 0.03 min, except for RMP (eluted 0.109 min earlier). The selectivity of the peak pair e/f suffered and the peaks began to merge with each other. The peak areas were affected more, averaging at ± 9%, especially for 3TC (area increased 19.3%). In conclusion, pH 3.0 does not provide as good selectivity as pH 2.5 or pH 2.0. The advantage of pH 2.5 is that RMP elutes faster and its area is slightly larger. According to Neue and Méndez [41], the retention changes between the molecule's ionised and non-ionised form depend weakly on the amount of organic solvent in the mobile phase and the retention changes between charged and uncharged form of the compound increases with increasing organic concentration.

The repeatability testing (Supporting Information Table S3) with statistical evaluation showed somewhat significant statistical differences ($p < 0.05$) or significant statistical differences ($p < 0.01$) between the known added concentration and the acquired concentrations with the applied method. The results varied depending on the compound: two compounds (SMX and CBZ) had no statistical difference between the added and acquired amounts, whilst most had some statistically significant differences between the added and recovered concentrations. These differences varied depending on concentration levels. CIP is the only compound with significant statistical difference (concentration level 1.0 mg/L). However, the different stock solution solvent (MilliQ-water) might explain the variation at this low concentration, even the recovery value with CIP at 1.0 mg/L is lower, which supports this interpretation. Yet, the RSD for CIP was <1.5%.

The recovery results for the proposed HPLC method (repeatability using stock compounds in either MeOH or MilliQ-water) from the intraday tests show that the recoveries varied between 90–108.8%. Variations in concentrations between different days can be explained up to a point by the possible instability of the old HPLC instrument. However, even though somewhat larger or lower recoveries were recorded, than what is recommended, the recoveries were accurate enough for the purpose of this method and thus acceptable.

The results of the inter- and intraday testing of the method for reproducibility are presented in Table 2. The overall retention time for each compound did not change markedly during interday analyses (standard deviations <± 0.01 min) except for RMP, whose standard deviation was 0.111 min.

The results for recovery testing are compiled in Table 2. The highest recovery for 3TC was obtained using 2% NH_4OH. The overall recoveries varied between 81.6–109.2% with RSDs between 0.1–5.3%. 3TC gave quite low recovery when using Strata-X sorbent: the best recovery was on 5 mg/L concentration, with a recovery of 43.3%. The collection of the 3TC-containing fraction separately was necessary to ensure at least somewhat adequate recovery. The problem is mainly because of the high polarity of 3TC (3TC also flushes out of the Kinetex XB-C_{18} column quite easily with $c > 100$ mg/L). Prasse et al. [13] have stated that because of their high polarity, the antiviral drugs are probably not effectively retained on SPE sorbent materials (see Table 1, $\log K_{ow}$ of 3TC is −1.29) and thus not on RP columns. The minimum requirement for analyte $\log K_{ow}$ for Strata-X is −0.5. According to Rummel [42], a general rule is that analytes with $\log K_{ow} < -1.0$ are very difficult to retain on many commercially available silica- or polymer-based reversed-phase chemistries. This is in accordance with our results.

In Table 2, the LOD and LOQ for the method using a urine matrix are presented. The LOD and LOQ for urine varied between 39–1610 and 129–5366 μg/L, respectively. The LOD as well as LOQ were higher for all compounds, except for NVP, in SPE-treated urine samples than in standard solutions, but not too high to prevent the utilisation of the method for the proposed purpose (detection from fresh urine samples with concentrations from tens to hundreds of mg/L).

The poor retention of 3TC causes problems when analysing water samples and with SPE while pre-treating urine. Different SPE elution methods were tested to find the optimum for all the compounds of interest: elution with 10% ACN, 50% ACN, 2% NH_4OH and 2% Acetic acid was tested. Of these 2% NH_4OH was selected. The 3TC fraction contained the interfering compounds from the urine sample that were washed away in this step. If 3TC was the main analyte of interest, it would be advisable to use another type of column and/or SPE sorbent that increases the retention of analytes with $\log K_{ow} < -1.0$. However, the goal was to develop a single method suitable for all the selected compounds that might occur simultaneously in urine.

To compare our results with Strata-X to other similar studies, some results from previous publications are collected in Table 3. A comparison of data is challenging since either the matrix or the SPE sorbent is not necessarily similar to our study; however, some generalisations can be made. In the case of TRI, ZDV, NVP and CBZ, the results are similar to other studies that have used Strata-X as sorbent [5, 30, 42, 43], though, in many cases the matrix has been human plasma [4, 44, 45]. For 3TC, the recovery obtained was better than ours, but the SPE method was optimised for antiviral pharmaceuticals. In that case [4], it was possible to adjust

Table 2. Reproducibility of the method, and recovery and LOD and LOQ of the method in urine matrix

		Compound							
		3TC	TRI	ZDV	CIP	NVP	SMX	CBZ	RMP
Interday[a]	*c* (mg/L)								
($n = 3$)	1	1.0 (0.05)	1.0 (0.003)	1.1 (0.02)	1.0 (0.04)	1.0 (0.02)	1.0 (0.02)	1.0 (0.02)	1.1 (0.06)
	10	10.3 (0.20)	10.2 (0.05)	10.0 (0.06)	10.1 (0.08)	10.3 (0.05)	10.2 (0.13)	10.2 (0.01)	9.8 (0.10)
	100	101.7 (1.56)	106.1 (0.63)	101.3 (0.24)	100.2 (0.22)	104.0 (0.50)	102.6 (0.48)	102.6 (0.12)	102.0 (0.30)
Intraday[a]	*c* (mg/L)								
($n = 3$)	1	0.9 (0.07)	1.0 (0.02)	1.1 (0.02)	1.0 (0.004)	1.0 (0.02)	1.0 (0.04)	1.0 (0.02)	1.1 (0.02)
	10	10.3 (0.12)	10.3 (0.18)	10.1 (0.07)	9.9 (0.15)	10.7 (0.52)	10.1 (0.17)	10.3 (0.17)	9.9 (0.19)
	100	102.0 (0.85)	105.9 (1.85)	102.0 (1.32)	99.5 (0.80)	105.6 (1.25)	104.8 (2.65)	105.0 (2.16)	102.3 (0.56)
Recovery[b] (%)	*c* (mg/L)								
($n = 3$)	5	43.3 (3.1)	98.7 (2.7)	99.4 (2.7)	109.2 (2.4)	99.4 (2.9)	96.0 (3.1)	97.8 (1.0)	90.7 (2.5)
	10	36.3 (3.0)	101.0 (1.4)	106.9 (0.5)	102.8 (3.6)	98.5 (3.4)	96.2 (5.3)	102.1 (3.3)	98.5 (2.6)
	25	17.6 (1.4)	101.0 (1.9)	93.2 (3.8)	81.6 (3.1)	105.0 (1.5)	83.0 (3.1)	97.8 (1.7)	105.8 (1.9)
	50	16.7 (1.0)	104.9 (0.2)	103.2 (0.4)	87.8 (0.5)	102.2 (0.1)	86.5 (0.7)	–	101.2 (0.7)
LOD urine[c]	(μg/L)	1610	39	189	189	71	115	41	503
LOQ urine[c]	(μg/L	5366	129	630	631	237	383	136	1676
t_R (min)[d]	Interday	0.490 (0.002)	1.917 (0.005)	1.978 (0.001)	2.038 (0.003)	2.333 (0.000)	2.450 (0.002)	2.908 (0.001)	3.548 (0.111)

a) Mean (±SD).
b) Mean (±RSD), analytes in same sample, 5–25 mg/L for CBZ.
c) Limits of detection and quantification of the method for urine matrix.
d) $n = 9$, mean (±SD).

Table 3. Comparison of recoveries obtained for the selected pharmaceuticals with different SPE sorbents and matrices

Compound	SPE sorbent	Matrix	Recovery (%)	References
Lamivudine (3TC)	Strata-X	plasma	96.8[a]	[6]
Trimethoprim (TRI)	Bakerbond C18	urine	90–98[b]	[30]
	Strata-X	water	97.6	[43]
	Strata-X	water	123	[7]
	BondElut Plexa	urine	70–77[b]	[29]
Ciprofloxacin (CIP)	Bakerbond C18	urine	45–83[b]	[30]
	SPE-C18	plasma	72.3	[46]
	3M-Empore MPC	urine	61.9	[45]
Zidovudine (ZDV)	Strata-X	plasma	98.1[a]	[6]
Nevirapine (NVP)	Strata-X	plasma	97.4[a]	[6]
	Bond Elute Certify	plasma	≥88	[44]
Sulfamethoxazole (SMX)	Bakerbond C18	urine	72–93[b]	[30]
	Strata-X	water	120	[7]
	BondElut Plexa	urine	86–90[b]	[29]
Carbamazepine (CBZ)	LiChrolut DYE	urine	105	[8]
	Oasis HLB	plasma	94–99.8[b]	[47]
Rifampicin (RMP)	Strata-X	plasma	55.8–72.9[b]	[25]

a) Method optimised for antiviral pharmaceuticals.
b) Recovery variation between different spiked concentration levels.

the sample in such way that 3TC was retained better in the sorbent. In this method, the focus was on recovering all the compounds simultaneously, which limited the sample pre-treatment options. For CIP and RMP, the results show better recoveries than that obtained in other studies (over 80% in our case, only up to 72% in other studies). Also, in the case of SMX, our results with Strata-X were better (96%) than in the previous study. However, the matrix is different in many cases. Overall, the results presented in Table 3 are in accordance with our recovery values.

4 Concluding remarks

A rapid LC method utilising new core–shell column technology was developed to screen three antiviral agents and

four antibiotics simultaneously from source-separated urine. Also, the reference compound CBZ was successfully analysed. Instrumental LOD and LOQ varied between 21–536 and 71–1786 μg/L, respectively, for eight standard compounds.

Recoveries obtained using Strata-X SPE-sorbent were good although some compromises were needed in order to be able to simultaneously analyse all eight compounds. Recoveries varied between 81 and 109% for all compounds except for 3TC (16–43%). In the case of 3TC, it is necessary to dilute the urine sample with concentrations over 5.0 mg/L to guarantee at least somewhat sufficient recovery. The recovery values using Strata-X are comparable with other previously published results. The LOD and LOQ for urine varied between 39–1610 and 129–5366 μg/L, respectively. However, while the LOD is somewhat high for urine samples, the method enables the detection of the selected pharmaceuticals from fresh urine samples in the relevant range of concentrations from ten to hundreds of mg/L.

The application of a core–shell column provides savings in both time and mobile phase consumption, since the reduced analysis time greatly compensates the increased flow rate of the eluent. Using two subsequent elutions from the same SPE tube saves sorbent material. The repeatability and recovery obtained using Kinetex XB-C_{18} column were in accepted ranges. The LOD and LOQ were low enough considering further uses of this method. The method offers an easy and practical way to study the concentration of the eight compounds belonging to therapeutic groups with different physicochemical properties.

If urine is used as a fertiliser, it should be ensured that the pharmaceuticals in human urine do not pose a threat to humans or to the environment. The developed method presented in this paper provides a useful tool for this. The method fits for the fast determination of selected pharmaceuticals from source-separated urine samples or wastewaters for further environmental risk assessment and degradation potential evaluation and enhances nutrient recycling from wastewater streams. It also helps to promote the safe fertiliser use of source-separated urine. The analytical protocol also enables the further use of this method in environmental and fertiliser use monitoring to promote sustainable sanitation practices and the use in laboratories that do not have MS detectors.

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Table S1. Results from the calibration with stock solutions

Peak	Analyte	Calibration range (mg L^{-1})	t_R (min)[a]	R^2	Equation (±SD of slope and intercept)	LOD[b] (µg L^{-1})	LOQ[b] (µg L^{-1})	*k'*	Selectivity between peaks
a	3TC	0.5-100	0.490 (0.003)	0.9999	y = 4.8365(±0.021)x-2.4327(± 0.823)	536	1786	0.8	$\alpha(a,b) = 7.5$
b	TRI	0.05-100	1.915 (0.002)	0.9992	y = 25.833(±0.278)x-9.3603(±9.614)	32	108	6.1	$\alpha(b,c) = 1.0$
c	ZDV	0.1-500	1.978 (0.001)	0.9997	y = 5.4836(±0.031)x-0.0058(±5.250)	66	661	6.3	$\alpha(c,d) = 1.0$
d	CIP	0.1-500	2.100 (0.004)	0.9999	y = 4.4385(±0.020)x-12.152(±3.755)	80	265	6.6	$\alpha(d,e) = 1.2$
e	NVP	0.05-250	2.336 (0.001)	0.9999	y = 13.042(±0.034)x-1.229(±2.945)	73	243	7.7	$\alpha(e,f) = 1.1$
f	SMX	0.1-250	2.446 (0.002)	0.9999	y = 11.399(±0.023)x-1.1715(±1.941)	106	353	8.1	$\alpha(f,g) = 1.2$
g	CBZ	0.05-100	2.908 (0.001)	0.9998	y = 26.667(±0.151)x-2.309(±5.373)	21	71	9.8	$\alpha(g,h) = 1.2$
h	RMP	1.0-500	3.483 (0.019)	0.9996	y = 2.8374(±0.019)x-7.8517(±3.561)	204	679	11.9	

[a]Mean (±SD), n = 9; [b]Instrumental detection and quantification limits when utilizing method presented in section "2.2 Liquid chromatography".

Table S2. Results of the robustness tests of the method: a) the effect of temperature, b) the effect of gradient change, c) the effect of lower eluent A starting percentage, d) the effect of different eluent pH

a	**30 °C**		**45 °C**	
Compound	t_R change (min)	Peak area change (%)	t_R change (min)	Peak area change (%)
3TC	0.013	-0.1	0.007	-5.3
TRI	0.014	-1.8	0.002	0.0
CIP	0.015	9.4	0.002	13.9
ZDV	0.014	5.4	0.001	6.3
NVP	0.002	1.1	0.002	-0.1
SMX	0.027	-0.6	0.003	-1.0
CBZ	0.013	0.7	0.003	0.9
RMP	0.002	-9.1	0.002	-5.2

b	**5** to70 % ACN	**5** to 85 % ACN		**5** to 60 % ACN	
Compound	Current method t_R (min)	t_R change (min)	Peak area change (%)	t_R change (min)	Peak area change (%)
3TC	0.488	0.005	4.1	0.005	-0.6
TRI	1.917	-0.078	0.8	0.0	-0.5
CIP	1.975	-0.079	-2.5	0.001	0.2
ZDV	2.037	-0.106	5.1	0.0	-0.8
NVP	2.333	-0.138	0.7	0.0	1.0
SMX	2.446	-0.149	0.9	0.0	-0.3
CBZ	2.906	-0.243	0.1	0.0	0.6
RMP	3.494	-0.368	-1.6	0.455	-0.7

c	**2** to70 % ACN		**2** to 85 % ACN		**2** to 60 % ACN	
Compound	t_R change (min)	Peak area change (%)	t_R change (min)	Peak area change (%)	t_R change (min)	Peak area change (%)
3TC	0.004	0.0	0.003	0.0	0.004	0.0
TRI	0.004	0.0	0.128	0.0	0.028	0.0
CIP	0.003	0.1	0.129	0.1	0.028	0.1
ZDV	0.003	0.1	0.113	0.1	-0.011	0.1
NVP	-0.001	0.0	0.103	0.0	-0.052	0.0
SMX	0.005	0.0	0.105	0.0	-0.061	0.6
CBZ	0.001	0.0	0.076	0.0	-0.175	0.0
RMP	-0.001	-0.1	0.044	-0.1	-0.321	-0.1

d	pH 2.0		pH 3.0	
Compound	t_R change (min)	Peak area change (%)	t_R change (min)	Peak area change (%)
3TC	0.013	0.0	-0.021	19.3
TRI	0.005	-2.3	0.004	2.0
CIP	0.009	0.3	0.010	3.2
ZDV	0.008	-0.2	0.004	3.3
NVP	0.120	6.5	-0.032	-8.9
SMX	0.027	-2.3	0.006	8.9
CBZ	0.008	7.2	0.009	5.4
RMP	-0.050	-1.2	0.109	3.0

The t_R change or peak area change refer to difference (either in minutes or percentages) compared to the proposed method in this article.

Table S3. Statistical comparison of the concentrations added and found with the developed method and method recovery

Intraday		**3TC**	**TRI**	**ZDV**	**CIP**	**NVP**	**SMX**	**CBZ**	**RMP**
***p*-values**[a]	*C* mg L^{-1}								
	1	0.535	0.394	< 0.05	< 0.01	0.343	0.517	0.187	< 0.05
	10	< 0.05	0.085	0.182	0.524	0.14	0.271	0.076	0.634
	100	< 0.05	< 0.05	0.125	0.395	< 0.05	0.087	0.057	< 0.05
Method recovery %[b]									
	C mg L^{-1}								
	1	90.0 (7.5)	101.2 (2.0)	108.3 (2.0)	96.6 (0.4)	98.7 (1.9)	101.8 (3.9)	102.0 (1.7)	108.8 (1.7)
	10	103.2 (1.2)	103.3 (1.7)	100.8 (0.7)	99.3 (1.5)	107.1 (4.8)	101.5 (1.7)	103.4 (1.7)	99.4 (1.9)
	100	102.0 (0.8)	105.9 (1.8)	102.0 (1.3)	99.5 (0.8)	105.6 (1.2)	104.8 (2.5)	105.0 (2.0)	102.3 (0.6)

[a]Null hypothesis: The concentrations added and recovered from samples are equal: $p < 0.05$ = almost statistically significant, $p < 0.01$ = statistically significant;
[b]Mean recovery (± RSD)

II

EFFECT OF SOURCE-SEPARATED URINE STORAGE ON ESTROGENIC ACTIVITY DETECTED USING BIOLUMINESCENT YEAST *SACCHAROMYCES CEREVISIAE*

by

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Effect of source-separated urine storage on estrogenic activity detected using bioluminescent yeast *Saccharomyces cerevisiae*

Sanna Jaatinen, Anniina Kivistö, Marja R. T. Palmroth and Matti Karp

Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

ABSTRACT

The objective was to demonstrate that a microbial whole cell biosensor, bioluminescent yeast, *Saccharomyces cerevisiae* (BMAEREluc/ERα) can be applied to detect overall estrogenic activity from fresh and stored human urine. The use of source-separated urine in agriculture removes a human originated estrogen source from wastewater influents, subsequently enabling nutrient recycling. Estrogenic activity in urine should be diminished prior to urine usage in agriculture in order to prevent its migration to soil. A storage period of 6 months is required for hygienic reasons; therefore, estrogenic activity monitoring is of interest. The method measured cumulative female hormone-like activity. Calibration curves were prepared for estrone, 17β-estradiol, 17α- ethinylestradiol and estriol. Estrogen concentrations of 0.29–29,640 μg L^{-1} were detectable while limit of detection corresponded to 0.28–35 μg L^{-1} of estrogens. The yeast sensor responded well to fresh and stored urine and gave high signals corresponding to 0.38–3,804 μg L^{-1} of estrogens in different urine samples. Estrogenic activity decreased during storage, but was still higher than in fresh urine implying insufficient storage length. The biosensor was suitable for monitoring hormonal activity in urine and can be used in screening anthropogenic estrogen-like compounds interacting with the receptor.

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Bioluminescence; estrogen; estrogenic activity; *Saccharomyces cerevisiae*; urine

1. Introduction

Natural and synthetic estrogens and their analogues have the ability to disrupt natural chemical processes in the wildlife, for example, by causing fish feminization.[1] Estrogens are excreted via human metabolism into urine mainly as conjugates (sulfate or glucuronide groups attached), which travel via the sewage to the wastewater treatment plants (WWTPs) as extensively diluted and mixed together with other types of wastewaters. Bacterial metabolism and enzyme activity can transform these conjugates back to their active forms in the sewers (before wastewaters enter the WWTP), [2,3] causing the distribution of estrogens into the receiving waters.[4] The concentrations in the WWTP effluents vary depending on the type of estrogen, from few to tens of ng L^{-1}.[3,5] Wastewaters arriving at WWTPs have been recognized as one of the main pathways via which endocrine disruptors, including estrogens, enter the environment. The degradation of estrogens in WWTPs is not yet fully understood.[6] The need for additional studies and cost-effective alternatives for the removal and degradation of estrogens from effluents has been noted.[7] The reduction of estrogen discharges to WWTPs could minimize their concentrations in effluents to inactive levels (concentration so low that no effect on environment can be detected;[8]). The main compound of interest regarding the environmental effects of estrogens has been synthetic 17α-ethinylestradiol, used in contraceptives, but also natural estradiol, estriol and estrone are of interest. Estrogen presence in WWTP effluents is primarily of domestic origin.[4] In addition, since estrogens may migrate into and in soils [9] from wastewater products used as fertilizers, measuring the concentration of estrogens prior to application is important. Thus, the monitoring of estrogenic activity of source-separated urine is of interest.

Separate collection of urine from other household wastewaters (source separation) is an environmentally feasible way to remove the source of human originated estrogens [4] from wastewater influents as well as to recycle the nutrients (N, P, K and micronutrients) which otherwise would burden the WWTP,[10] thus reducing the treatment requirements. Urine comprises approximately 1% of the wastewaters arriving at municipal WWTPs, but contains in liquid form 60% to 90% of N, P and K a person ingests,[11] as well as many micronutrients. Thus, source-separated urine is a good alternative fertilizer, which adds major key nutrients into soil (e.g.

CONTACT Sanna Jaatinen sanna.jaatinen@tut.fi

phosphorus [12]). Source separation promotes decentralized sanitation concepts which have received more attention [13] as the need for sustainable fertilizer sources has been recognized, and source separation is practiced, for example, in Sweden,[14] Germany [15] and Australia.[16] Source-separated urine should be stored for 6 months to achieve the required hygienic level regarding pathogens [17] and afterwards it can be used in fertilization (for discussion on using human excreta on plant production, see Ref. [18]). During storage, urine typically undergoes ureolysis where urea is transformed to ammonia and consequently pH is raised greater than nine, leading to pathogen inactivation. Application of sheep urine has been shown to enhance the leaching of radiolabelled estrogens in soils compared with application with distilled water.[19] In addition, estrogens have been shown to de-conjugate in soil.[20] If estrogens are not degraded already during storage, they can cause harmful environmental effects when leached in ground waters. Thus, estrogens should be removed and/or degraded from urine before fertilizer applications to mitigate their effect on the environment.

In order to use source-separated urine in crop fertilization, the use of a biosensor becomes a convenient method for overall estrogenic activity detection to demonstrate that the estrogenicity has diminished from the urine. In this study, a genetically modified yeast *Saccharomyces cerevisiae* was used.

Yeast cell biosensors have been regarded as a viable alternative for first-stage screening of endocrine disrupting compounds since they are easy and fairly inexpensive to perform [21]; they have high specificity to hormones due to the lack of endogenous steroid receptors,[22,23] and can be grown on media devoid of steroids.[24] Yeast cells are more resistant to environmental contaminants compared with mammalian cells, which make them advantageous for use in measuring complex environmental samples.[21] Many applications have utilized an extra-chromosomal reporter system with β-galactosidase as a substrate-based reporter protein (yeast estrogen screen, YES [25]), human breast cancer estrogen-sensitive MCF-7 cells in E-SCREEN assay [26] and estrogen-receptor mediated chemically activated luciferase gene expression (ER-CALUX assay [27]). Androgens have been detected from urine using yeast with a modified androgen receptor.[22,23,28] The application of yeast estrogen (and androgen) bioassay to calf urine using green fluorescent protein required extensive pre-treatment steps involving solid phase extraction and the use of organic solvents.[29,30] However, mainly the aforementioned studies have used a human androgen-receptor (male hormone) based strain, whereas this paper focuses on the human estrogen-receptor strain.

To our knowledge, no information is available on using a bioluminescent yeast biosensor to monitor the estrogenic activity of source-separated human urine. The objective of this study was to demonstrate that a bioluminescent yeast cell-based biosensor can be applied as a rapid and simple screening tool in the overall estrogenic activity detection from source-separated urine with and without storage without laborious pre-treatments. The bioavailability of the estrogens was studied by adding β-glucuronidase to the samples, as estrogens are mainly excreted as conjugates. The aim was to use the biosensor as a screening tool for studying estrogenic presence in the samples in order to gain knowledge on the safety of urine in agricultural use, as well as to demonstrate the suitability of the biosensor in urine and environmental sample monitoring as a fast, stand-alone analytical tool.

2. Materials and methods

2.1. Chemicals and materials

Yeast nitrogen base w/o amino acids was purchased from Difco, agar-agar from Merck, and D-glucose, adenine, L-tryptofan, L-leucine, L-histidine and D-luciferin were obtained from Sigma-Aldrich. Estrone (E_1, ≥99%), 17β-estradiol (E_2, ≥98%), 17α-ethinylestradiol (EE_2, ≥98%) and estriol (E_3, ≥97%) were purchased from Sigma and used as standards for estrogenic activity measurements. Sterile Octavia-water was provided by Fresenius Kabi Ag (Germany). MilliQ-water (18.2 mΩ cm conductivity, Millipore) was used in media preparation. Urease (jack bean urease, lyophilized 5 U mg^{-1}, *EC* 3.5.1.5) was obtained from Merck and ionic strength adjusting (ISA) solution was purchased from Thermo-Scientific. *S. cerevisiae* strain BMAEREluc/ERα with modified estrogen receptor alpha (ERα) was developed by Leskinen et al. [21]. Liquid synthetic dextrose medium (SD-medium), AHL-medium (containing essential amino acids adenine, L-histidine, L-leucine aka AHL-medium) and AHL-agar were prepared according to Välimaa et al. [31].

2.2. Yeast cultivation

S. cerevisiae BMAEREluc/ERα was cultivated in AHL-agar. It was grown for 48 h at 30°C and subsequently one colony was transferred into 5 mL of liquid medium (two replicates) which was incubated at 30°C overnight and 300 rpm shaking. Un-inoculated pure AHL-medium was used as control. For bioluminescence and hormonal activity assays, yeast cells were grown to an optical

density (OD_{600}) of 3.0 and subsequently the culture was diluted with fresh AHL-medium to OD_{600} of 0.8 and used in the assay.

2.3. Hormonal activity assay

Bioluminescence assay was performed according to the method described by Leskinen et al. [21]. The method was tested for each estrogen (stock solutions of 10 mM in EtOH of E_1, E_2, EE_2 and E_3) by analyzing concentrations from 0.027 ng L^{-1} to 29.6 mg L^{-1} (in 10% EtOH), in order to create standard curves. The analyses were done in triplicate with 10% EtOH as a reference blank.

A standard curve was constructed by pipetting 10 µL aliquots of hormones into white 96-well plates (Thermo Electron Corporation). As blank, 10 µL 10% EtOH was used. Aliquots of 90 µL of the diluted yeast culture (BMAEREluc/ERα) were then added into the wells containing the samples. The plates were incubated at 30°C for 2.5 h at 300 rpm. After incubation, 100 µL aliquots of sterile filtered 1 mM D-luciferin in 0.1 M Na-citrate buffer (pH 5.0) were pipetted into the wells and bioluminescence was measured immediately using Victor^2 Wallac 1420 Multilabel counter (PerkinElmer Life Sciences, Inc.).

2.4. Characterization of the matrix

The major constituents of fresh and stored human urine (as indicators of storage; described in more detail below) were determined in order to characterize the urine matrix. pH and conductivity of both types of urine samples were measured with Orion SA 720 pH-meter and WTW LF95, respectively.

The sum of ammonium (NH_4^+) and ammonia (NH_3) was determined with an ammonia selective, gas-sensitive membrane electrode (Thermo-Orion), which measures the NH_3 concentration. ISA solution was added to the samples and standards to convert ammonium to ammonia. Urea concentration was determined by measuring ammonia from the sample, then adding the urease enzyme and afterwards letting the sample stand for ca. sixteen hours in room temperature, while urease catalyzed urea decomposition to ammonia. Subsequently, ammonia was measured and the difference between the two ammonia concentrations equalled the amount of urea in the sample.

Ion chromatography (Dionex DX120) was used to determine the anions of interest (PO_4^{3-}, Cl^-, SO_4^{2-}, NO_3^-, NO_2^-) from the urine. The separating column was AS9-HC (Dionex) equipped with pre-column AG9-HC (Dionex). The eluent used was a buffer prepared of 12 mM Na_2CO_3 and 5 mM $NaHCO_3$. Urine was diluted with MilliQ water (1:100 v/v). The diluted urine was filtered (nylon 0.45 µm, VWR). Further analysis steps were made according to standard SFS-EN ISO 10304-1:en.[32]

For the determination of cations, an atomic adsorption spectrophotometer was used. Na, K, Mg and Ca were analyzed from urine with AA200 analyzer (PerkinElmer) using air acetylene flame. Before analysis, urine samples were filtered (0.45 µm nylon, VWR). The filtered samples were diluted at ratios from 1:50 to 1:5000 with MilliQ-water using acid washed glassware. The dilution for each sample depended on the cation and nature of urine. A 0.18 M CsCl + $LaCl_3$ solution (Merck) was added to the samples of which Ca was analyzed to stop the formation of substances that inhibit Ca atomization. The analysis was done according to Finnish Standard Association (SFS) standards 3018 [33] and 3044.[34]

Determination of dissolved organic carbon (DOC) was conducted according to SFS-EN standard 1484.[35] Urine samples were diluted with MilliQ-water and filtered through 0.45 µm prewashed syringe filter (nylon, VWR), after which the samples were analyzed with a TOC-5000 Analyzer (Shimadzu).

2.5. Detection of estrogenic activity in urine

For hormonal activity testing, triplicate urine samples collected from a volunteer female taking oral contraceptives, a female not using synthetic hormones, and male urine (two volunteers), child urine (both a boy and a girl), mixed urine (a combination of the previous) and stored urine (mixed, 1 or 5 months in polypropylene containers, in dark at room temperature) were used. The collected urine samples were not spiked with estrogens, but analyzed as such. The time points of 1 and 5 months were selected in estrogenic activity testing to obtain an understanding about the kinetics of hormone-like activity and degradation. As blank, 10 µL 10% EtOH and 10% EtOH in urine were used. Aliquots of 10 µL of samples were pipetted in triplicate without any pre-treatment into a white 96-well plate and analysis performed as described earlier. Analysis was repeated thrice for each urine sample. Concentrations of 0.027, 2.72, 272.4 and 27,238 µg L^{-1} of E_2 were spiked in fresh and stored urine to test the storage effect, and the recovery was determined against the E_2-standard in 10% EtOH. Adaptations of the sensor for a different matrix were not needed,[21] emphasizing the fact that the yeast biosensor can be used without any pre-treatment of the sample as a stand-alone tool in monitoring estrogenic activity changes during urine storage.

The bioavailability of the estrogens was tested either by heat treating samples at 80°C for 30 min (which is a normal procedure to hydrolyze compounds) or by

hydrolyzing them with β-glucuronidase enzyme (β-D-glucuronoside glucuronosohydrolase from *Escherichia coli* K12, *EC* 3.2.1.31, Fisher Scientific) at 46°C for 45 min to break the conjugated bonds. β-Glucuronidase interacts with the glucuronide group attached to the main molecule and breaks the bond, thus releasing the original estrogen in its bioactive form. Before hydrolysis with β-glucuronidase, pH of the samples was adjusted to 6.0–6.5 with 0.1 M NaOH or 0.1–1.0 M HCl. As a reference, the same samples were incubated at 46°C for 45 min without β-glucuronidase addition. The samples were heated in water baths at the specific temperatures, left to cool to room temperature and consecutively analyzed as previously described.

The impact of urine (fresh and stored) filtration on biosensor performance was not tested, as filtration/sterilization is not feasible in practice on a large scale and thus not considered feasible for applicability as a screening tool to assess urine safety in agricultural use. Filtration would be time-consuming and the costs would be high, as well as all the bacteria responsible for estrogenic degradation would be removed.

2.6. Analysis of obtained data

Light emission was measured as relative light units (RLU). By normalizing the RLU values of the samples with the background bioluminescence of the sensors, the data obtained from different experiments and/or luminometers can be compared.[21] The induction of the sensor by the sample was calculated as fold induction (FI): $FI = SL_S/SL_B$, where SL_S is the average bioluminescence of the yeast sensor (measured from three replicates) and SL_B is the bioluminescence of blank solvent (at least four replicates). FI is a dimensionless unit. FI in *y*-axis was plotted against $\log_{10}$ of the estrogen concentration. The limit of detection (LOD) describes the lowest measurable concentration and/or signal of the method. The minimum response considered as positive (FI_{LOD}) was determined according to Long and Winefordner [36]: $FI_{LOD} = 2(X_B + 3SD)/X_B$, where X_B is the average background bioluminescence value of the sensor (four 10% EtOH blanks included in each assay) and SD is the standard deviation. The LOD was subsequently determined from the standard curve using FI_{LOD}.

To evaluate the average estrogen concentration in urine samples, an average estrogen calibration curve was constructed based on four estrogens and their average molecular weight (281.8 g mol^{-1}). Although in some cases the results were below FI_{LOD}, an estimation of the estrogen concentration has yet been made. Of course, it has to be kept in mind that the real urine samples possibly contained other estrogenic compounds which also induce a signal and thus the calculated estrogen concentrations represent the concentration of all estrogen-like compounds in the sample. For FI values higher than the average estrogenic response curve, the calibration curve of EE_2 was used (biosensor gave the best signal, as described later).

3. Results and discussion

The goal of this study was to investigate the applicability of the bioluminescent yeast cell-based biosensor as a fast, stand-alone analytical screening tool in estrogenic activity monitoring in urine. In the present study, the urine samples required no pre-treatment (e.g. filtration, extraction or centrifugation). The method could be applied to urine as such.

3.1. Characteristics of the matrix

Source-separated urine contains amino acids, solids, salinity and inorganic and organic constituents (urea, creatinine, acids, etc.).[37] The measured characteristics of human urine are presented in Table 1. Fresh urine usually has pH ranging from 5.5 to 6.5, as described in this study. Urine contains several grams per liter of DOC (7.3 g L^{-1}) and urea (4.9 g L^{-1}), a compound which transforms into ammonia during storage due to bacterial ureases.[38] Urine contains macronutrients (N, P and K) in relatively large quantities (g L^{-1}), whereas micronutrients up to tens of mg L^{-1} (Table 1). Therefore, removing urine from wastewater streams not only reduces the organic load arriving at WWTPs, but also enables nutrient recycling. The collected urine can be used as a liquid fertilizer, while WWTP effluent is too diluted in nutrients and contains more pathogens due to the presence of fecal matter. The composition of urine changes during storage: organic matter degrades,

Table 1. Characteristics of the urine matrix.

Urine composition (unit)	Fresh	Stored
pH	5.8	9.4
Conductivity (mS cm^{-1})	9.1	25.9
DOC (g L^{-1})	7.29	2.81
$NH_4^+ + NH_3$ -N (g L^{-1})	0.57	8.67
Urea (g L^{-1})	4.95	1.05
Anions		
NO_3^- (g L^{-1})	0.16	n.d.
SO_4^{2-} (g L^{-1})	1.61	1.28
PO_4^{2-} (g L^{-1})	2.86	1.18
F^- (mg L^{-1})	91.6	55.8
Cl^- (g L^{-1})	2.67	2.40
Cations		
Na (g L^{-1})	0.83	1.01
K (g L^{-1})	0.52	1.17
Mg (mg L^{-1})	76.0	n.d.
Ca (mg L^{-1})	88.0	3.1

Note: n.d. = not detected.

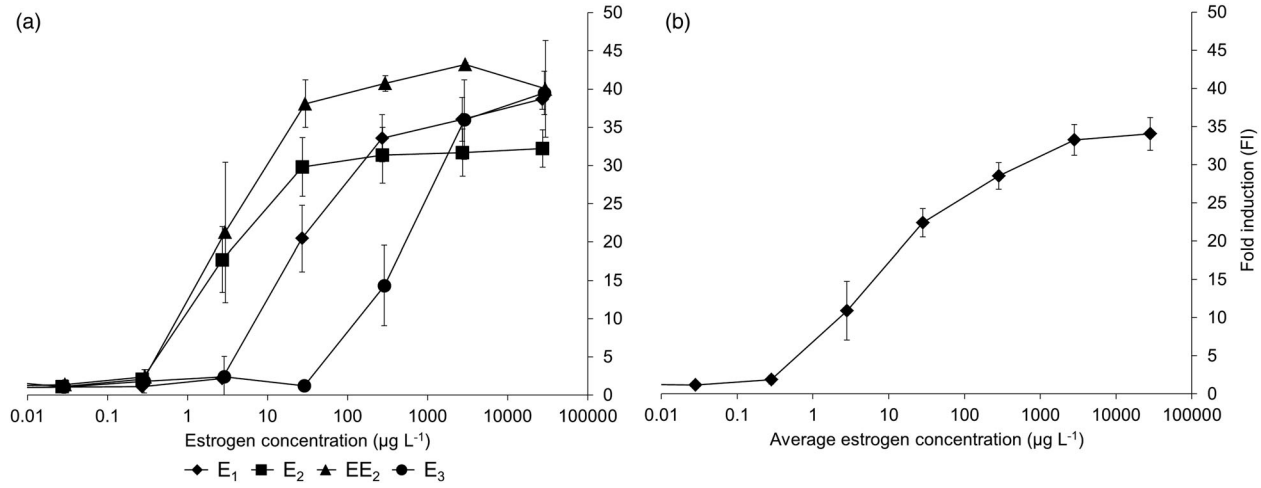

Figure 1. (a) Standard curves for estrone (E_1), 17β-estradiol (E_2), 17α-ethinylestradiol (EE_2) and estriol (E_3). (b) A standard curve calculated as the average of four estrogens from Figure 1(a) for the estimation of estrogenic concentration. The error bars represent the standard errors from three measurements. Estrogenic concentration determination: $FI < FI_{LOD}$: estimated from average estrogen curve; $FI > FI_{LOD}$: calculated from average estrogen curve; FI > average estrogen curve FI: calculated from EE_2 standard curve.

Mg and Ca salts precipitate (e.g. as struvite) and the salt content of urine decreases as a result of precipitation. After storage, the pH increased from 5.7 to 9.0–9.5 and urea transformed into ammonia.

3.2. Hormonal activity assay

Results obtained from constructing standard curves for estrogens (E_1, E_2, EE_2, E_3) in 10% EtOH were as presented in Figure 1(a). All the estrogens had similar standard curves, with an exponential increase in the signal at a specific concentration range. Similarly to Salste et al., [39] who studied WWTP effluents with the same biosensor, the yeast biosensor produced a signal with respect to all of the studied estrogens in a concentration-dependent way, thus demonstrating the repeatability of the results produced by this particular yeast strain in a different laboratory and matrix. The yeast response to estrogens varied depending on the estrogen (Figure 1(a)) with repeatable results, and mainly the response was obtained with estrogen range from 0.29 to 29,640 µg L^{-1}. In order to estimate the overall estrogenic concentration, an average of four estrogens was taken (Figure 1(b)) to approximate concentrations in urine.

The highest response with the biosensor was obtained with the synthetic EE_2. The FI_{LOD} for the biosensor was 2.4, corresponding to approximately 2.8 µg L^{-1} E_1, 0.28 µg L^{-1} E_2, 0.29 µg L^{-1} EE_2 and 35 µg L^{-1} E_3. The LOD determined from the average concentration curve corresponded to 0.29 µg L^{-1} estrogens. A comparison of different yeast cell-based bioassays and their detection limits to the current bioassay is presented in Table 2. A detailed comparison of the biosensors is unequal as some of the studies have measured estrogen equivalents, while some have used the bioassay only to demonstrate estrogenic activity: in those cases concentrations have been measured with chromatographic methods. One must bear in mind that practically all of the previous biosensor analyses used sample enrichment before analysis if the matrix has been some other than a standard solution (e.g. EtOH). Overall, the LOD obtained in this study is practical in monitoring real urine samples and the analysis is relatively fast to perform. In addition, as chromatographic methods usually require sample preparation and clean-up before analysis, use of this biosensor is well justifiable in simple and rapid monitoring. Compared with the analysis using mass spectrometer, which normally has LOD of few ng L^{-1} (e.g. Ref. [4]), the obtained LODs are higher; but compared with conventional liquid chromatography in combination with urine sample pre-treatment which has LOD around 0.1–0.2 µg L^{-1}, the results are comparable.[40]

Table 3 shows the typical excretion amount of estrogens per day in urine. The average amount of urine produced is ~1.2 L person^{-1} d^{-1},[37] which dilutes the estrogen concentrations in collected urine. Common birth control pills contain approximately 30 µg of EE_2. When comparing the LOD of the yeast biosensor with these excretion concentrations, it is obvious that the sensitivity of the sensor is applicable in urine estrogenic activity monitoring, especially, as the sensor reacts to other estrogenic compounds as well.

Estrogenic concentrations spiked in fresh and 5-month stored urine were different from the standards (0.027, 2.72, 272.4 and 27,238 µg L^{-1}). The results are presented in Figure 2(a),(b). The spiked E_2 concentrations in

Table 2. Comparison of yeast cell-based bioassays.

Bioassay	Reporter protein	Matrix	Sample pre-treatment	Time needed for analysis	LOD (μg L^{-1})	Reference
S. cerevisiae BMAEREluc/ERα	Luciferase	Pure compound in 10% EtOH	Not needed	2.5 h Incubation	0.008 (E_2)[a]	Leskinen et al. [21]
S. cerevisiae (yEGFP)	Green fluorescent protein	Spiked calf urine	Solid phase extraction, de-conjugation	De-conjugation overnight, plate drying overnight, 24 h incubation	1.0 (E_2 and EE_2)[a]	Bovee et al. [29]
S. cerevisiae	β-galactosidase	Pure compound in EtOH	Not needed	Three-day incubation	0.0015 (E_2)	Routledge and Sumpter [28]
Yeast estrogen screen (YES)	β-galactosidase	River water	Solid phase extraction	Pre-treatment 120–150+ min, 3-day incubation	0.0001 estrogen equivalents (EE_2)	Vermeirssen et al. [46]
S. cerevisiae BMAEREluc/ERα	Luciferase	Wastewater effluent	Solid phase extraction	Solid phase extraction 20+ min, freeze drying 4.5 h, chromatographic fractionation 28 min, LC–MS/MS[b] 15 min 2.5 h incubation	LOD was not determined for yeast assay	Salste et al. [39]
S. cerevisiae BMAEREluc/ERα	Luciferase	Pure compounds in 10% EtOH	Not needed	2.5 h Incubation	2.8 (E_1), 0.28 (E_2), 0.29 (EE_2), 35 (E_3)	This study
		Human urine (real samples)	De-conjugation	De-conjugation 45 min, 2.5 h incubation	0.38-3,804 (measured overall estrogenic concentration)	This study

[a]Measured as nM, converted by the authors.
[b]LC–MS/MS = liquid chromatography–mass spectrometry.

fresh urine gave reasonable signals, while the FI values were lower than that in standards prepared in 10% EtOH (0.5–6.2 FI units). This might be explained by sample toxicity, which might have had an effect on the yeast, thus decreasing the bioluminescence. However, the reason for such spiked E_2 behavior is not crucial regarding the use of the biosensor to the suggested application. The spiked E_2 concentrations in stored urine had high signals, with recovery comparable to fresh urine, if standard error is taken into account. This could be due to the purity of the estrogens, as they were not glycosylated, thus making them more bioavailable. The urine blank induced quite high signal, which caused a low FI value in stored urine. A high urine blank signal in stored urine is explained by the release of estrogenic compounds in urine in addition to the presence of other estrogenic substances.

Leskinen et al. [21] have already proven that yeast estrogen strains have behaved and responded to hormones (two androgens, E_2) and xenoestrogens similarly with yeast estrogen screen. Relative estrogenic potencies determined with their assay seemed comparable to those determined using *in vitro* bioassays. Urine always contains estrogens (even in urine from males); thus, having urine blank is not possible. Even though long enough stored urine might be devoid of estrogens, the composition of the sample would be different and it might still contain estrogen-like compounds that induce a signal with the biosensor. The use of a different matrix as blank seems inappropriate, for example, as a solution of ammonia or water is not comparable with the composition of and the matrix effect caused by urine. However, urine blank is not needed since all FI values are corrected by dividing with the blank signal, and the aim of this study was not to determine exact concentrations but to monitor the overall change in estrogenic activity, in which the biosensor performed accordingly.

Table 3. Typical estrogen concentrations in human urine and their excretion.

Estrogen	Excretion in urine (μg d^{-1})	Excretion as glucuronides [47] (%)	Excretion as free form [47] (%)
Estrone (E_1)	0.54–54[a] 3.2–16.1[b]	85–89	1–3
Estradiol (E_2)	0.54–16.3[a] 4.6–269[b] 10.3[c]	90–95	1–3
Ethinylestradiol (EE_2)	7.1[b]	90–95	n.a.
Estriol (E_3)	1.4–43.2[a]	90–95	0–2

Note: n.a. = not available.
[a]U. Turpeinen, Helsinki University Central Hospital Laboratory, Helsinki, Finland, personal communication.
[b]Ref. [43], 269 μg E_2 d^{-1} for pregnant women.
[c]Average concentration in human urine [48].

3.3. Estrogen bioavailability

The bioavailability of the estrogens and the response of the biosensor are affected by the presence of estrogens as conjugates in urine (Table 3); thus, breaking up the conjugated bonds should improve the sensitivity of the sensor strain and provide knowledge on the magnitude of de-conjugation potential in soil.

Figure 3(a),(b) describes the signal given by the yeast biosensor in different urine samples without and after different treatments (Table 4). As previously described, time points of 1 and 5 months were selected in estrogenic activity testing due to the availability of samples. The overall recommended time for urine storage is 6 months,[17] but shorter periods are commonly used.

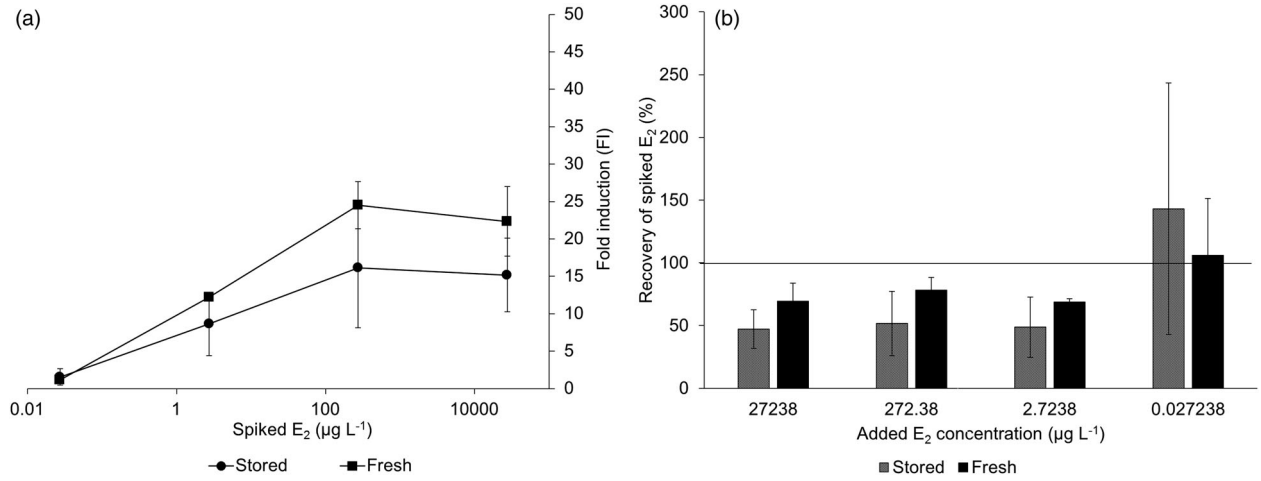

Figure 2. Comparison of the storage effect with spiked samples. Fresh urine was collected on the previous day. Urine (mixed) was stored for 5 months in plastic jars, at room temperature. Samples were spiked with four E_2 concentrations and analyzed. The FI values represent the mean of three replicates, with error bars showing standard error. (a) The higher FI values for fresh urine are explained by the lower bioluminescence of the blank. The blank stored urine had a higher background signal, thus decreasing the overall FI values. (b) Recovery of the added E_2 concentration compared with the E_2 standard in 10% EtOH. With fresh urine, the recovery was better.

Only unconjugated forms of estrogens contributed to the ERα activity when urine was measured without treatment (first and second bar), and the results were repeatable. Thus, bioavailability testing was conducted to get the response for all estrogenic activities present in the sample (measuring the sum of free and glucuronidated forms). Bioavailability of estrogens is important as different soil microorganisms [41,42] may break down the conjugated bonds, thus releasing active hormones in the soil.

Heat treatment at 80°C for half an hour resulted in increased activities in urine from a female not taking contraceptives (FI value 18.5, 14 µg L^{-1}) and a female child (FI value 2.5, 0.35 µg L^{-1}) when compared with urine without heating (concentrations below average LOD, 0.05–0.29 µg L^{-1}). The addition of β-glucuronidase and incubation at 46°C for 45 min increased the FI values of all urine samples, ranging from FI 7.8 (1.4 µg L^{-1}) to FI 41.4 (10,200 µg L^{-1}, a female without contraceptives, determined using standard for EE_2), except for the

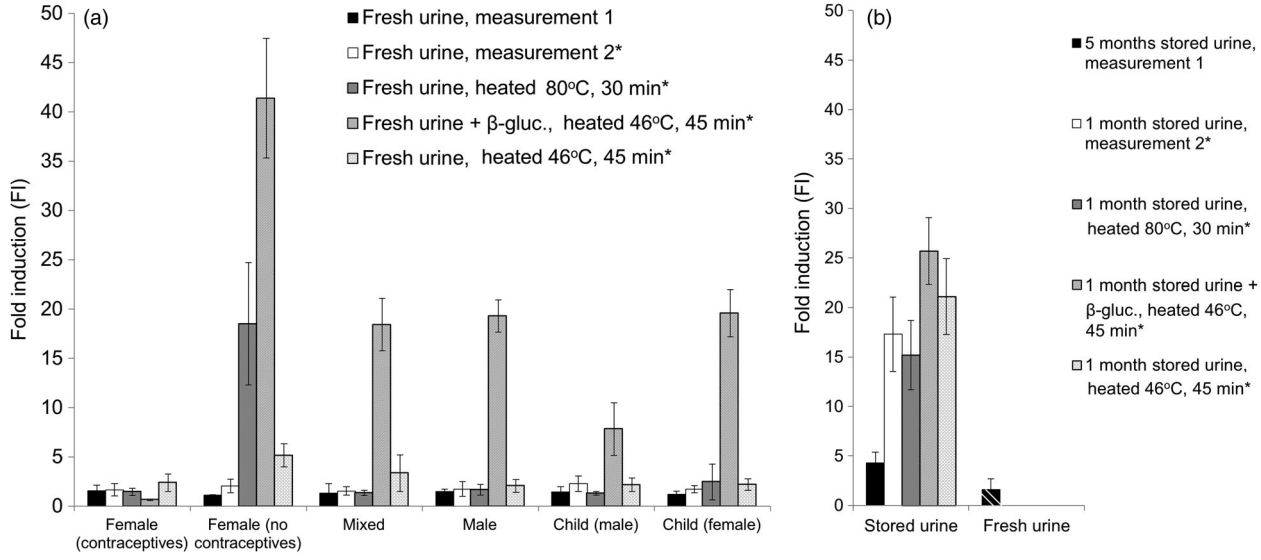

Figure 3. FI of different urine samples using the bioluminescent yeast biosensor. The bars represent the mean of three replicates, with error bars showing standard error. β-gluc. = β-glucuronidase. (a) FI values for the different fresh urine samples with and without different pre-treatments. (b) FI values of fresh and stored urine (at room temperature in plastic jars with a tight lid, 1 or 5 months) with and without different pre-treatments. * indicates that urine samples were collected and analyzed at the same time (measurement 2).

Table 4. Different pre-treatments conducted to urine samples presented in Figure 3.

Figure		Pre-treatment
3A: Fresh urine	Measurement 1: fresh urine only	None
	Measurement 2: fresh urine with different pre-treatments, indicated by*	None
		Heat treatment 80°C (30 min)
		Heat treatment 46°C (45 min)
		Heat treatment with β-glucuronidase addition 46°C (45 min)
3B: Stored urine	Measurement 1: Stored urine (5 months)	None
	Measurement 2: Stored urine (1 month) with different pre-treatments, indicated by*	None
		Heat treatment 80°C (30 min)
		Heat treatment 46°C (45 min)
		Heat treatment with β-glucuronidase addition 46°C (45 min)

female taking contraceptives (FI 0.6, <LOD). Mere incubation at 46°C did not affect the FI values as much as enzyme addition, except in the case of the female taking oral contraceptives and in stored urine. The high FI value for the female not taking contraceptives can be explained with the menstrual cycle and/or pregnancy: during different phases, the excreted estrogen concentrations can vary markedly.[43] The observed signals corresponded to estrogen concentrations of 0.27–2,964 μg L^{-1} (determined from the standard curves based on measured FI, Figure 1(a),(b)), the highest of which could be due to other estrogen-like compounds excreted in urine and binding to the ERα receptor.

Estrogen excretion for men [43] has been calculated to average 3.2 μg d^{-1}, whereas our results using heating and β-glucuronidase addition gave 15 μg L^{-1}, which adjusted to 1.2 L d^{-1} makes 18 μg d^{-1}; however, this estrogen concentration includes all estrogen-like compounds excreted in male urine. Clearly, the method's sensitivity was better with β-glucuronidase addition: the samples which earlier gave lower FI values now showed more clearly estrogenic activity. This was in accordance with the results published by Kaiser et al. [44] who tested their yeast biosensor with swine urine and noted that samples needed a pre-treatment before analysis to break the conjugated bonds (incubation with *Helix pomatia* solution), because estrogen conjugation reduced the method's sensitivity. The FI values were higher than the calculated LOD, except in the case of the female taking contraceptives, which could not be explained.

The effect of urine storage on estrogenic activity was as hypothesized. Storing urine affected the bioavailability of estrogens. After 1 month of storage, mixed urine gave a markedly higher signal (FI value 17, 9.5 μg L^{-1}) than fresh urine (FI value 1.4, 0.1 μg L^{-1}, <LOD). Urine stored for 5 months gave an FI value of 4.3 (6.2 μg L^{-1}), indicating that some biotransformation had occurred. This was expected since source-separated urine is never devoid of bacteria and as previously stated, filtration of urine on a large scale is not feasible due to economic reasons. The bacterial enzymatic (glucuronidases, estradiol dehydrogenases and sulfatases) and metabolic activity breaks the conjugated bonds, thus increasing the observed signal, which reduces over time due to estrogen degradation. After 5 months, the estrogenic concentration was still higher than in fresh urine (0.1 μg L^{-1}, <LOD), indicating the presence of estrogen-receptor binding compounds. According to the review by Yu et al. [7] some microorganisms isolated from the intestine can convert E_2 to E_1 and vice versa, or degrade E_1, E_2 and E_3; they can also co-metabolize EE_2 in the presence of other estrogens. Thus, further studies are required to monitor how the estrogenic activity changes and which enzymes are active during the 6-month storage period. Furthermore, it might be interesting to add certain microbial mixed communities known to degrade estrogens to the urine.

The advantage of biosensors in estrogenic activity monitoring is their capability to estimate the cumulative estrogenic effects of a variety of chemicals in an environmental sample [8] that correspond to the overall activity of all ERα-binding chemicals present.[45] Although the measurement of exact concentrations is informative, measuring the overall estrogenic activity can be more useful regarding practical applications, such as fertilizer use. The yeast used in this study gave a positive response to all major forms of estrogenic compounds and it could also be used in pre-screening anthropogenic estrogen-like compounds which interact with the receptor, such as bisphenol-A or parabens (e.g. Ref. [21]). To be more specific, the method applied in this experiment measures the cumulative concentration of endocrine disruptor compounds (EDCs), not just estrogens. Therefore, it is very difficult to make comparisons to other methods, as the molecular diversity of such EDCs is so vast that it is impossible to measure the accurate concentrations of a single compound. In addition to the before mentioned, it is possible that degradation products of EDCs are inducing a signal. As stated previously, the calculated estrogenic concentrations in this study were only estimates when calculation was based on average estrogen molecular mass.

Biosensors are also suitable for the development for onsite tests because of their high specificity. Since estrogens have been shown to migrate in soil, this screening tool could be used to test estrogenic activity in soil samples after extraction to liquid. This method may

also prove useful in a quick screening of endocrine-disrupting chemicals for the efficacy of municipal WWTPs, which has been previously performed, for example, by Desbrow et al. [4] using a different reporter gene as well as a selection of sample pre-treatment steps.

Contrary to the conventional chromatographic methods, the method has several advantages, including complementarity to traditional analytical chemistry analyses, a minimal sample pre-treatment need, the provision of analyte bioavailability information and simultaneous analysis of multiple samples. The bioluminescent yeast assay is easy to handle, robust, cost-efficient and needs no high-tech analysis equipment but can be used with cheap portable detectors.

4. Conclusions

To our knowledge, this is the first experiment where bioluminescent yeast biosensor *S. cerevisiae* with β-glucuronidase addition has been used to monitor estrogenic activity in source-separated urine. From the results obtained in this study, we concluded the following:

- Estrogenic activity present in urine after storage implied that the recommended length of urine storage may be insufficient in reducing the overall estrogenic activity to agriculturally 'safe' levels.
- Four estrogens (E_1, E_2, EE_2 and E_3) could be detected using the biosensor with an effective measuring range of 0.29–29,640 μg L^{-1} and LOD corresponding to 0.28–35 μg L^{-1} of estrogens.
- The yeast sensor produced a cumulative signal, which included both natural and synthetic estrogens and estrogen-like compounds.
- The addition of β-glucuronidase and heating for 45 min at 46°C enabled the assay to be used in monitoring the change in the estrogenic activity of source-separated urine.
- The yeast sensor can also be used in screening anthropogenic estrogen-like compounds which interact with the ERα receptor.
- The method has several advantages on conventional chromatographic techniques, for example, a minimal need for sample pre-treatment and the simultaneous analysis of multiple samples in a short three-hour analysis time.

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Disclosure statement

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III

THE EFFECT OF URINE STORAGE ON ANTIVIRAL AND ANTIBIOTIC COMPOUNDS IN THE LIQUID PHASE OF SOURCE-SEPARATED URINE

by

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The effect of urine storage on antiviral and antibiotic compounds in the liquid phase of source-separated urine

Sanna T. Jaatinen[a], Marja R.T. Palmroth[a], Jukka A. Rintala[a] and Tuula A. Tuhkanen[a,b]

[a]Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland; [b]Department of Biology and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

ABSTRACT

The behaviour of pharmaceuticals related to the human immunodeficiency virus treatment was studied in the liquid phase of source-separated urine during six-month storage at 20°C. Six months is the recommended time for hygienization and use of urine as fertilizer. Compounds were spiked in urine as concentrations calculated to appear in urine. Assays were performed with separate compounds and as therapeutic groups of antivirals, antibiotics and anti-tuberculotics. In addition, urine was amended either with faeces or urease inhibitor. The pharmaceutical concentrations were monitored from filtered samples with solid phase extraction and liquid chromatography. The concentration reductions of the studied compounds as such or with amendments ranged from less than 1% to more than 99% after six-month storage. The reductions without amendments were 41.9–99% for anti-tuberculotics; <52% for antivirals (except with 3TC 75.6%) and <50% for antibiotics. In assays with amendments, the reductions were all <50%. Faeces amendment resulted in similar or lower reduction than without it even though bacterial activity should have increased. The urease inhibitor prevented ureolysis and pH rise but did not affect pharmaceutical removal. In conclusion, removal during storage might not be enough to reduce risks associated with the studied pharmaceuticals, in which case other feasible treatment practises or urine utilization means should be considered.

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1. Introduction

The global target for sustainability and resource efficiency has raised the issue of developing the use of urine especially as a fertilizer for crop production,[1] as urine contains macronutrients (N, P, K) in relatively high quantities. Urine comprises 60–90% of N, P and K ingested by a person in liquid form,[2] in addition to many micronutrients. The present fate of urine ranges from discharges to the sewage, for example, in most cities with a modern wastewater treatment system, to conditions where urine is excreted into a hole in the ground where it infiltrates through the soil. However, in order to better manage the use of urine, the source separation of urine is increasingly studied and proposed as a method for promoting sustainable nutrient management. Source-separated urine can replace a large portion of nutrients applied in agricultural fields [3] and new opportunities are opening as a nutrient source, for example, in microbial electrochemical technologies [4] and microalgal cultivation.[5] Source-separated urine is typically stored before agricultural fertilizer use, and, for example, six months storage is recommended by World Health Organization [6] to remove pathogens from urine before use in fertilization purposes.

However, urine may contain pharmaceuticals as in humans they are to a great extent excreted in urine, which may be disadvantageous to its use, and, for example, in countries without proper sanitation, pharmaceuticals often end up straight in the environment. Various types of pharmaceuticals are used globally. Many studies have focused on compounds commonly associated with the high standards of living (painkillers, lipid modifiers, etc.) and the potential antibiotic resistance of bacteria at wastewater treatment plants (WWTPs). Their fate has been studied at WWTPs and receiving waters (for a review, e.g. [7]), while to our knowledge, pharmaceutical behaviour during long-term storage (e.g. six months) has not been sufficiently studied. Pharmaceuticals, which have been a little studied, are antivirals, which are commonly used as a combination in human immunodeficiency virus (HIV) treatment (lamivudine, zidovudine and nevirapine). [8–10] Antibiotics (trimethoprim and sulfamethoxazole) are used in combination with selected antivirals;[11] as

CONTACT Sanna T. Jaatinen sanna.jaatinen@tut.fi Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

well as antibiotics (ciprofloxacin and rifampicin) that are used to treat tuberculosis (referred to as anti-tuberculotics). Latent tuberculosis may be activated during HIV-infection which is why these pharmaceuticals are often used together, especially in developing countries. These pharmaceuticals have not been investigated as extensively although they are administered in large doses (hundreds of mg/d), but, for example, sulfamethoxazole, trimethoprim and ciprofloxacin have been detected in WWTP influent and effluents [12,13] (antivirals are practically not at all studied). It is not possible to obtain the real yearly consumption data globally, but the consumption goes hand in hand with the amount of confirmed HIV infections.

The behaviour of pharmaceuticals during storage is an important factor to take account in order to minimize risks when planning urine re-use options, such as fertilizer applications. During the storage of urine, pharmaceutical concentration reduction could occur either through precipitation and/or adsorption, but also via chemical and biological pathways, the latter indicated by increased pH and thus bacterial activity. During the storage, urea in urine, typically in a few months, undergoes ureolysis (mainly due to bacterial urease enzymes) where urea transforms into ammonia with a simultaneous increase in pH.[14] The increased pH may result in ammonia volatilization thus decreasing the fertilizing value of urine. Urine contains bacteria from the urinary tract, and typically those originating from faeces, which is commonly present as contamination;[15] thus, bacteria may be responsible for pharmaceutical degradation during storage. However, from a practical point of view, whether the compounds are biologically degraded or remain in the storage vessel is not very crucial.

To study the effect of ureolysis and thus storage pH on pharmaceutical removal, an urease-inhibiting compound can be added into the urine, and subsequently urea transformation into ammonia is avoided. The treatment prolongs the fertilization impact and decreases the loss as volatilized ammonia. The prevention of ureolysis also prevents the pH rise in the urine, which might prevent the decay of bacteria at high pH that might play a role in the behaviour of pharmaceuticals during urine storage. A urease-inhibiting agent nBPT [*N*-(n-butyl)-thiophosphoric triamide] was found to decrease ureolysis.[16] Thus, testing its effect on microorganism survival via lowering the urine pH is of interest, as well as its effect on pharmaceutical behaviour.

The aim of this study was to evaluate the effect of urine storage of six months on selected pharmaceuticals, which were studied as divided into three therapeutic groups referred to as antivirals, antibiotics and anti-tuberculotics, and as individual compounds with no amendments. The effects of faecal contamination and urease inhibitor to affect the compounds' behaviour in urine were also examined. The results obtained from this experiment can be used to evaluate the safety aspects of urine re-use options.

2. Materials and methods

2.1. Urine, chemicals and materials

Urine samples were collected from eight healthy volunteers, both male and female, receiving no medication, while the initial pharmaceutical concentrations in collected urine were not investigated. The samples were stored in 0.5–1.0 L, sterilized polypropylene jars for a maximum of one day (in 4°C) before they were all combined and carefully mixed in a volumetric flask, after which homogenized urine was divided into 150 mL portions in experimental jars. The adsorption onto polypropylene jars was assumed to be negligible, since polypropylene does not adsorb, for example, carbamazepine, trimethoprim or sulfamethoxazole.[17]

The eight studied pharmaceuticals (United Corporation Ltd., Kenya) were antivirals lamivudine (3TC), zidovudine (ZDV) and nevirapine (NVP); antibiotics trimethoprim (TRI) and sulfamethoxazole (SMX); and anti-tuberculotics ciprofloxacin (CIP) and rifampicin (RMP), which belong to three therapeutic groups, referred to as antivirals (3TC/ZDV/NVP), antibiotics (TRI/SMX) and anti-tuberculotics (CIP/RMP). Carbamazepine (CBZ) was included in this experiment as a well-studied recalcitrant pharmaceutical reference compound.

Urease inhibitor nBPT dissolved in water-soluble organic solvent was received as a ready-made StabilureN®-solution (Agra Group, Czech Republic). Faeces were mixed with deionized water (MilliQ-water, Millipore; 18.2 mΩ cm conductivity) to produce a stock concentration of 10 $g_{fresh\ weight}$/L. Autoclaving, which is often used to prevent biological activity, of the control was not performed, since when tested it changed the composition of urine by transforming urea into ammonium.

The stock solutions of pharmaceuticals, 5.0 g/L, were prepared in methanol, except CIP in MilliQ-water due to low solubility in methanol. CBZ (antiepileptic) stock solution (in methanol) was 1 g/L. Stock solutions of pharmaceuticals were added in the sample jars to produce concentrations (10–80 mg/L) which could be present in source-separated urine originated from HIV and tuberculotic patients (see Table 1). These concentrations were calculated using typical pharmaceutical concentrations used per person per day as described in WHO database, [18] and as the literature indicates 2.7–70% of the administered dosages of different pharmaceuticals (Table 1)

Table 1. The administered dosage (per person per day) for the selected pharmaceuticals in this study, their approximate excretion percentages as a parent compound and the corresponding amount excreted into urine (per 1.5 L) per day.

Compound	Typical dosage (mg d^{-1})[a]	Excretion to urine % (unchanged)	Amount excreted in urine (mg L^{-1} d^{-1}) (1.5 L urine d^{-1})	Amount spiked in urine samples (mg L^{-1})
Lamivudine (3TC)[b,c]	300	70.0	140	50
Zidovudine (ZDV)[d]	600	16.0	64	80
Nevirapine (NVP)[e,f]	400	2.7	7	10
Ciprofloxacin (CIP)[e]	1000	45.0	300	50
Rifampicin (RMP)[e]	600	15.0	60	70
Sulfamethoxazole (SMX)[e]	320	20.0	43	50
Trimethoprim (TRI)[e]	1600	44.0	469	50
Carbamazepine (CBZ)[g]	1000	3.0	20	10

[a][18], [b][19], [c][20], [d][21], [e][22], [f][23], [g][24].

are excreted in urine. Thus, the used concentrations illustrate conditions when pharmaceutical-containing urine is not excessively diluted with urine containing no pharmaceuticals.

2.2. Experimental set-up

The experiment consisted of the following four different storage assays with pharmaceuticals, each performed in triplicate (Figure 1): (1) eight pharmaceuticals were stored separately and (2) in three therapeutic groups (antivirals, antibiotics and anti-tuberculotics), which were stored as such and with amendments of (3) faeces (final concentration of 9 mg/L, an average concentration shown to be present in source-separated urine [15]) and (4) urease inhibitor (final nBPT-concentration of 2 mM, [manuscript in preparation]). In addition, urine was stored as a control as such or with amendments, but no pharmaceuticals were added (Figure 1). All assays were performed as triplicates and altogether 60 jars were amended with 150 mL urine (jars closed tightly with a lid) and were stored for six months in the dark at room temperature.

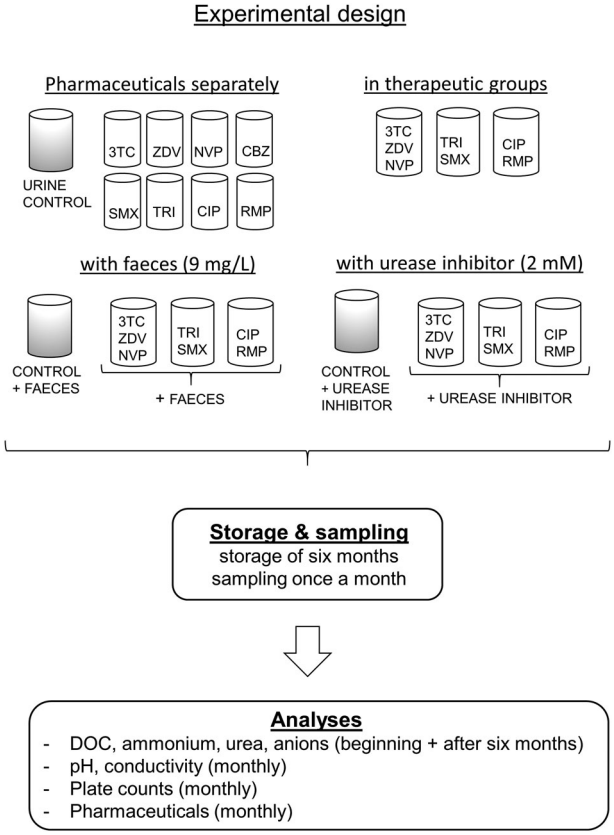

Figure 1. Schematic of the experimental design. Pharmaceuticals were added into urine as separate compounds and in therapeutic groups as such and with different amendments. All 20 experiments were performed as triplicate resulting in 60 experimental jars.

The jar lids were opened once a month when samples were taken thus resulting in the O_2-depletion in the jar head space and generating anoxic conditions before next sampling. Sample volume was 3 mL and the total volume of samples taken out during the experiment was 21 mL per jar. The effect of volume reduction due to volatilization in the jars was presumed to be small due to tight lids.

2.3. Analyses

Before measurements and sampling (3 mL) the jars were shaken vigorously a few times. The shaking of the sample did not have an effect on the precipitates (remained in the jars) and all samples taken for further studies were filtrated (0.2 µm Nylon filter, VWR) before analysis. pH and conductivity were measured from the sample jars; pH with WTW 3210 pH-meter with a SenTix 41 pH electrode and conductivity with WTW 3210 conductivity meter with a Tetraflon 325 electrode.

The temperature was monitored with Data Loggers (MSR Electronics GmbH, Switzerland) and data were analysed with MSR software (V5.12.04). The temperature was 19.5 ± 0.5 °C, (except peaks for 2–3 hours at 25°C during five days) during six months.

Dissolved organic carbon (DOC) was determined according to an SFS (Finnish Standard Association)

standard.[25] Urine samples, diluted with MilliQ-water and filtered through 0.45 µm prewashed syringe filter (Nylon, VWR), were analysed with TOC-5000 analyser (Shimadzu).

Anions (PO_4^{3-}, SO_4^{2-}, NO_3^-, NO_2^-) were determined with ion chromatography (Dionex ICS-1600) from filtered (0.45 µm nylon syringe filters, VWR) samples according to standard SFS-EN ISO 10304–1:en.[26] The separating column used was IonPac AS4A-SC anion exchange column with ASRS-300 suppressor (2 mm). The eluent was a buffer containing 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$.

The sum of ammonium (NH_4^+) and ammonia (NH_3) nitrogen concentrations was determined with an ammonia-selective electrode (Thermo-Orion). The electrode was calibrated with the standards of 100 and 1000 ppm solutions of (NH_4^+) − *N* from ammonium chloride. The ionic strength-adjusting solution (Thermo-Orion) was added to the samples and standards to convert ammonium to ammonia. The gas-sensitive membrane electrode measured the NH_3 concentration of the sample. Urea concentration was determined by measuring ammonia from the sample, then adding the urease enzyme (jack bean urease, *EC* 3.5.1.5, Sigma-Aldrich), and afterwards letting the sample stand for ca. 16 hours in room temperature, while the urease catalysed urea decomposition to ammonia. Subsequently, ammonia was measured and the difference between the two ammonia concentrations equals the amount of urea in the sample.

For the analysis, 3 mL of filtrated (0.2 µm Nylon filter, VWR) sample was taken, of which 1 mL was pre-treated for analysis and the rest was stored in the freezer (−18° C) for LC-ESI-MS/MS. Pharmaceuticals were determined from filtrated samples, which were pre-treated with solid phase extraction (SPE) and analysed with Hewlett-Packard Agilent 1100 HPLC with UV detection using a method optimized for the simultaneous detection of all eight compounds, described by Pynnönen and Tuhkanen.[27] They also presented the method suitability for the urine matrix and the recoveries for the present pharmaceuticals as well as limit of detection (LOD) and quantification (LOQ) values (Table 4). The effect of sample pH change on retention to SPE sorbent/HPLC column was taken into account by testing pharmaceutical retention to the SPE sorbent in different pHs (6–9) and constructing correction factors for different pHs based in compound peak areas (data not shown here). This was done as pH of the urine samples for HPLC-analysis was not adjusted as the addition of pH-adjusting agent into small sample volumes would have caused sample dilution that should have been accounted for. Monthly, the data from chromatographic separation regarding each urine treatment were studied and concentrations for pharmaceuticals were calculated according to previously prepared external standards. The HPLC-UV chromatograms were investigated to evaluate the behaviour of the compounds during storage by studying the parent compound peaks and screening for peaks with similar spectra as parent pharmaceuticals. Qualitative LC-ESI-MS/MS analysis (manuscript in preparation) with the same pre-treatment as described for HPLC-UV were used to evaluate possible transformation products from assays as well as to verify the concentration of the parent compound.

3. Results

Behaviour of eight pharmaceutical compounds during the six-month storage of urine spiked with individually, in therapeutic groups (antivirals, antibiotics and anti-tuberculotics and with different amendments (faeces and urease inhibitor) was studied using CBZ as a reference pharmaceutical. In addition, urine was stored as such (Table 2) and with amendments. In all assays, precipitates, either solid or both solid and floating, were visible after six months at the bottom of the jars, and in some cases already earlier.

pH and conductivity were followed as indicators of biological and ureolytic activity. During the six-month storage, the pH rose up to 8.7–9.6 in urine as such and with individual pharmaceuticals and in the groups of antivirals and antibiotics while with group of anti-tuberculotics pH remained lower (7.9) (see Table 3). In the presence of faeces, pH rise was similar or lower than without, while the presence of the urease inhibitor mitigated pH rise in all three groups as well as in urine control resulting in final pH 7.4–8.2. Changes in conductivity followed in general, changes in pH: when pH rose (up to 9) conductivity rose from initial ca. 9 up to 20–25 mS/cm, while with assays where pH was lower (6.5–8.2) conductivity remained lower as well (<15 mS/cm) (data not shown).

The pharmaceutical concentrations in the liquid phase were analysed once a month in all assays during

Table 2. Characteristics of fresh and six months stored urine.

Parameter (unit)	Fresh urine[a]	Stored urine[a]
pH	6.3 (0.0)	9.6 (0.0)
DOC (g/L)[b]	3.9 (0.0)	1.5 (0.0)
$NH_4^+ + NH_3$ -N (g/L)	0.26 (0.02)	1.04 (0.03)
Urea (g/L)	0.89 (0.05)	0.05 (0.03)
Conductivity (mS/cm)	9.1 (0.0)	25.9 (0.3)
NO_3^- (mg/L)[b]	32.4 (5.6)	n.d.
NO_2^- (g/L)[b]	0.04 (0.02)	0.77 (0.07)
PO_4^{3-} (g/L)[b]	0.93 (0.26)	0.35 (0.02)
SO_4^{2-} (g/L)[b]	1.61 (0.0)	0.91 (0.02)

Note: n.d. = not detected.
[a]mean (± stdev), $n = 3$.
[b]filtrated samples.

Table 3. pH after six months of storage. Data are mean (±standard error), $n = 3$.

		pH							
		Separately		Therapeutic groups		Faeces		Urease inhibitor	
		Start	6 months	Start	6 months	Start	6 months	Start	6 months
Antivirals	3TC	6.3 (0.0)	9.3 (0.2)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	8.2 (0.1)
	ZDV	6.3 (0.0)	9.6 (0.0)						
	NVP	6.3 (0.0)	9.5 (0.0)						
Antibiotics	TRI	6.4 (0.0)	8.7 (0.4)	6.4 (0.0)	9.2 (0.2)	6.2 (0.0)	7.8 (0.8)	6.5 (0.0)	7.4 (0.0)
	SMX	6.3 (0.0)	9.4 (0.2)						
Anti-tuberculotics	CIP	6.3 (0.0)	8.9 (0.1)	6.4 (0.0)	7.9 (0.2)	6.2 (0.0)	7.4 (0.2)	6.5 (0.0)	7.7 (0.5)
	RMP	6.3 (0.0)	9.4 (0.1)						
Reference	CBZ	6.4 (0.0)	9.6 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Control	6.3 (0.0)	9.6 (0.0)	6.3 (0.0)	9.6 (0.0)	6.1 (0.0)	9.5 (0.0)	6.5 (0.0)	7.7 (0.0)

Note: n.a. = not available.

the six-month storage (Table 4). Sampling was performed monthly as it was speculated that not much difference can be observed in a shorter time span. In all assays and with all amendments RMP concentration reduced gradually during six months (Figure 2) below the LOD while the other pharmaceuticals including CBZ (Figure 2, shown as an example) exhibited 23–52% removals and 3TC even up to 76% when incubated alone or as therapeutic groups (Table 4). Faeces amendment resulted in a similar or lower (even less than <1%) reduction of the pharmaceutical concentrations than without faeces. Urease inhibitor reduced the removal of all pharmaceuticals except with CIP (Table 4).

The HPLC-UV chromatograms of monthly samples of all five assays were screened for peaks with similar spectra as parent pharmaceuticals to assess the presence of the initial pharmaceuticals and/or transformation compounds. For RMP, whose concentration reduced to below LOD, no parent compound peak was detected in the chromatograms (with or without different amendments) after six months (small parent compound peak was still present after five-month storage), which indicated its complete removal from the liquid phase as seen in Figure 2. As for 3TC, NVP, ZDV and TRI, no potential transformation product peaks were detected in chromatograms. On the contrary, anti-tuberculotic CIP (Figure 3(a)) and antibiotic SMX (Figure 3(b)) both produced a peak with almost similar a spectrum to the parent compound, after three to five months depending on amendment (additional peaks indicated with an arrow) suggesting degradation.

In addition to HPLC-UV, a qualitative LC-ESI-MS/MS technique was used to screen pharmaceuticals from the assays of individual compounds after six-month storage. The removal of RMP was confirmed while also four unidentified transformation products were observed in assays with RMP. In addition, various transformation products for CIP, SMX and 3TC were identified, while not any transformation products for NVP, ZDV and TRI (available in the literature) were detected (Table 4).

The qualitative analysis showed no marked differences between different amendments. For 3TC, one degradation product was found in three amendments while CIP had seven identified transformation products, of which one was observed in every amendment. In the case of SMX, one degradation product was seen in every amendment.

4. Discussion

The present results show that storage of urine-containing pharmaceuticals for six months varies depending on the compound and concentration reductions in the liquid fraction of urine range from small to marked

Table 4. Reduction of pharmaceuticals in the liquid phase after six months of storage. Data are mean (±standard error), $n = 3$. 'Individually' refers to just one pharmaceutical amended in urine.

		Reduction (%) in the liquid phase after six months				Transformation products detected	
	Pharmaceutical	Individually	Therapeutic groups	Faeces	Urease inhibitor	HPLC-UV	LC-ESI-MS/MS
Antivirals	Lamivudine (3TC)	75.6 (7.8)	51.4 (8.3)	28.9 (22.3)	<1	n.d.	+
	Zidovudine (ZDV)	51.5 (3.7)	45.6 (0.5)	<1	<1	n.d.	n.d.
	Nevirapine (NVP)	25.6 (6.2)	28.8 (3.1)	24.5 (2.9)	16.9 (5.0)	n.d.	n.d.
Antibiotics	Trimethoprim (TRI)	23.7 (1.7)	40.3 (4.8)	42.0 (3.6)	18.9 (1.6)	n.d.	n.d.
	Sulfamethoxazole (SMX)	24.0 (4.7)	32.2 (3.0)	<1	<1	+	+
Anti-tuberculotics	Ciprofloxacin (CIP)	51.1 (10.6)	41.9 (27.4)	38.5 (8.5)	44.2 (19.5)	+	+
	Rifampicin (RMP)	>99	>99	>99	>99	n.d.	+
Reference	Carbamazepine (CBZ)	26.8 (3.5)	n.a.	n.a.	n.a.	n.d.	+

Notes: For pharmaceuticals in urine [27]; LOD: 3TC 1.6 mg/L, ZDV 189 µg/L, NVP 71 µg/L, TRI 39 µg/L, SMX 115 µg/L, CIP 189 µg/L, RMP 503 µg/L, CBZ 41 µg/L. LOQ: 3TC 5.4 mg/L, ZDV 630 µg/L, NVP 237 µg/L, TRI 129 µg/L, SMX 383 µg/L, CIP 631 µg/L, RMP 1.7 mg/L, CBZ 136 µg/L. + = transformation product detected. n.d. = not detected. n.a. = not available.

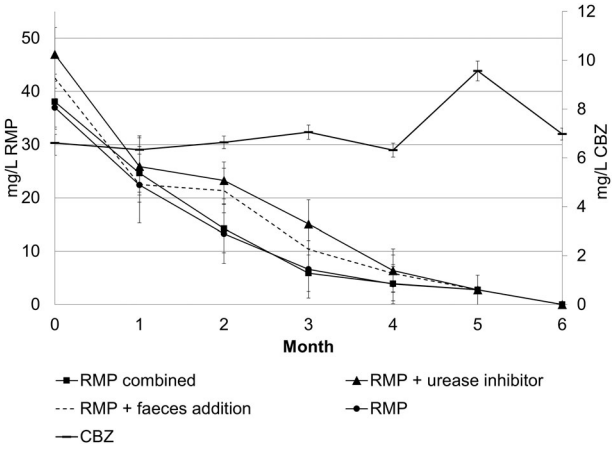

Figure 2. The concentration of rifampicin (RMP, left y-axis) and carbamazepine (CBZ, right y-axis) in monthly samples during the six-month storage of urine with different amendments. Combined refers to the results of urine amended with therapeutic groups. The error bars represent the standard error between three replicates.

(from 23% to 75%) while RMP was removed >99% in all assays. Furthermore, the potential for the formation of transformation products, which can be also harmful, is evident during storage. The concentration reductions were lower with faeces amendment and the lowest with urease inhibitor amendment. The results showed marked reductions for some compounds but under the studied conditions neither biological nor chemical mechanisms occurred to enable the complete removal of the studied pharmaceuticals from the liquid phase (except RMP). Thus, it appears that conditions in source separation and storage of urine do not favour the concentration reduction and six-month storage as recommended by WHO does not mean a complete removal of the studied pharmaceutical risks. Therefore, methods to actively decrease pharmaceutical concentrations in the source-separated urine should be studied to enable its use.

Although source separation of urine is not a new concept, there are currently only a few studies available on pharmaceutical behaviour during urine storage,[28–30] none of which has used the same pharmaceuticals as the current study, except for CBZ. The concentration reduction during 3–4-month storage of urine for CBZ was reported to be 20–80% [28–30] and the highest reductions for several pharmaceuticals (up to 80–90%) were observed during pH-controlled storage (3–4 months [28]). It was discovered indicating that a low pH facilitates concentration reduction during urine storage. However, it was acknowledged that the length of the storage period (3–4 months) was not enough to completely remove the pharmaceuticals,[28] a finding which concurred with the results presented in the current study. In the current study, room temperature and six-month storage were used, but parent compounds were still present in urine afterwards. Varying storage periods (from three months to a year), different pHs (from pH 2 to 11) and temperatures (from 4°C to 38°C) have been tested, and somewhat marked effects on pharmaceutical concentrations have been discovered,[28–30] while complete removal has not been observed. In a modern wastewater treatment system, urine is flushed to WWTPs along with the pharmaceuticals, where conjugated pharmaceuticals can transform back into their active forms by microbial metabolism and be transported to watersheds; the transformation can take place already in the sewer network (pressurized sewer in anaerobic conditions, retention time 21 h [31]) where CIP was slightly degraded while SMX, TRI and CBZ had negative removal implying a microbial or

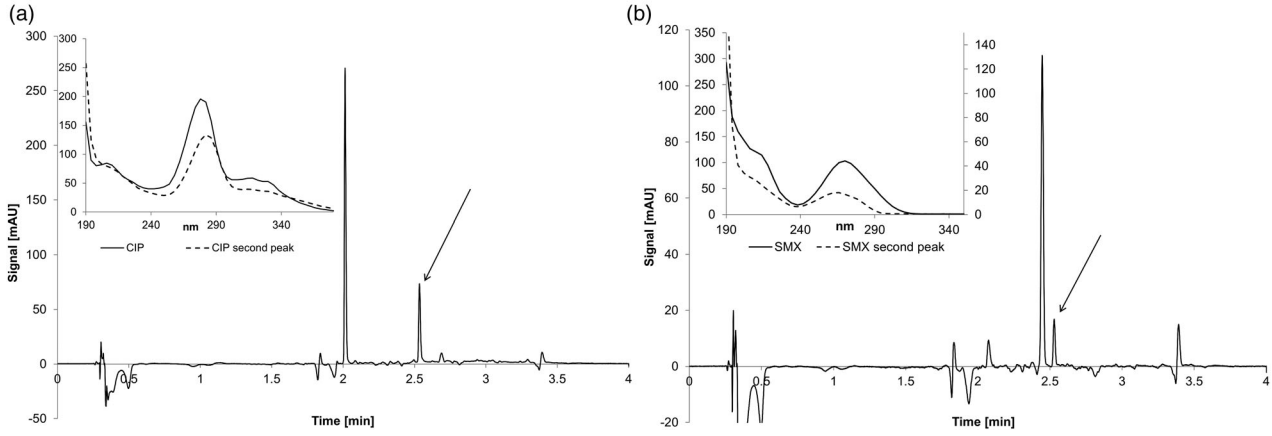

Figure 3. Chromatograms and spectra of CIP (a) and SMX (b) after five months of storage of urine-amended individual pharmaceutical. The additional peaks are indicated with arrows. The spectra of the additional peaks have resemblance to the original compound. Note that the signal of SMX second peak is on the secondary axis.

chemical breakdown of conjugated compounds.[31] Source separation of urine removes this route of pollutants. Yet, urine storage might not be enough to reduce pharmaceutical risks in fertilizer use in which case, other feasible treatment practices to enhance pharmaceutical removal should be studied.

The complete removal of RMP, also with the two studied amendments, was strikingly opposite to the other seven studied pharmaceuticals. The main mechanism for the removal of RMP from the liquid phase could be biodegradation, which would be a novel finding as no information of RMP biodegradation could be found. Biological removal was proposed, but not confirmed, by the disappearance of the RMP parent compound and appearance of transformation products in the LC-ESI-MS/MS analysis. Chemical degradation also produces transformation products which cannot be ruled out. However, as RMP is a very large molecule (823 g/mol) compared with the other studied pharmaceuticals (a range from 224 to 331 g/mol [27]), this is opposite to the assumption that the smaller the molecule, the easier it would be to degrade it. It might be, that although pH with RMP alone rose >9, the different bacteria present in urine thrive at different stages and are able to break down the compound gradually. While RMP was similarly removed also in the presence of CIP, and where pH remained 6.5–7.9 in all assays, the explanation could be that urine contained bacteria that are resistant to RMP/CIP but might not have ureases thus causing lower pH. The disappearance of RMP in the presence of all amendments suggested that it is readily biodegradable.

The concentrations of the seven other pharmaceuticals reduced somewhat less indicating that neither biological nor chemical processes were sufficient in enabling the complete removal of the compounds. The effect of antivirals and antibiotics can be seen in the pH of the different urine assays: with antivirals, pH rose close to or over 9 despite the urease inhibitor addition whereas with antibiotics pH rise remained lower (except for TRI/SMX and SMX individually, pH > 9.2). As the pH of samples amended with faeces were similar with urine amended with the urease inhibitor, it is possible that in the urease inhibitor assays the main affecting factor in the pH was the urease inhibitor together with pharmaceuticals. Therefore, in assays with faeces the combination of two antibiotics and anti-tuberculotics was probably still sufficient in preventing bacterial growth. The reason for the low or no (bio)degradation could therefore also be the concentrations of antibiotic compounds or the inhibitory effects on microbes present in urine, as antibiotics are designed to prevent bacterial growth. The inhibitory effect of therapeutic groups on bacterial growth was seen, for example, as lower pH rise with CIP and RMP, of which CIP is, in addition to tuberculosis treatment, effective against common bacteria found in urine, such as *Proteus mirabilis*, *Escherichia coli* and *Klebsiella* sp.[32] The aforementioned bacteria are also susceptible to TRI and SMX.[33] In the case of antivirals, the high concentration could lead to toxicity, but the antivirals ought not to affect the bacteria as they are designed to stop virus infections.

Pharmaceuticals are excreted in urine in tens to hundreds of mg/L (see Table 1), while in WWTPs the influent typically contains ng/L to µg/L concentration of pharmaceuticals. Although these concentrations are orders of magnitude lower than in urine, the bacteria in WWTPs are not able to break down all of the pharmaceutical compounds and addition to that, transformation products are formed, which together with pharmaceuticals end up in the environment.[12] As inhibition is concentration dependent, it is likely that the expected lower pharmaceutical concentration in real-life urine storage systems might enhance biological degradation; however, no information is available on the toxicity response of the studied pharmaceuticals. On the other hand, the emergence of transformation products in the LC-analysis suggested the (bio)transformation of the pharmaceuticals and the emergence of breakdown products in the LC-ESI-MS/MS analysis implied the formation of possibly more harmful products from paired compounds. The environmental relevance of transformation products is yet quite a new field of study.[13] The degradation products and their formation are currently studied). Further determination and identification of the formed compounds will give indication regarding the possibility of the compounds having more harmful characteristics than the main compound. The removal of the antibiotic compounds could be expected to some extent in large-scale source-separated urine, but the behaviour of the antiviral pharmaceuticals may prove more problematic.

The current study showed that at least some of the pharmaceuticals selected in this study underwent degradation although only slightly, and the degradation could either be chemically or biologically induced. Earlier studies of pharmaceutical biodegradation using closed bottle tests and lower pharmaceutical concentrations have demonstrated that CIP (test concentration 5.95 mg/L [34]), SMX (3.8 mg/L [35]) and TRI (0.5 mg/L [36]) are not readily biodegradable. ZDV and NVP are also recorded as not readily biodegradable (degraded 3–4%), the same as 3TC, which have been found to pose toxic effects on activated sludge (degradation −3%) (all compounds 50 mg/L [37]). However, closed bottle tests use low bacterial density: source-separated urine can

support the growth of bacteria up to about 10^8 cfu/mL,[38] while in closed bottle tests the recommended bacterial concentration ranges from 10^4 to 10^6 cfu/L.[39] The current study used pharmaceutical concentrations of 10–80 mg/L which are similar or higher than in previous tests, yet the bacterial density in urine was probably quite high. Thus, the biodegradability rate of these compounds may have increased with higher bacterial densities and the diversity of microorganisms in urine could have increasingly affected the biodegradability, as it was probably with SMX, which has been shown to be degradable by bacteria in WWTPs (89% removal [12]). Biological activity was proposed by pH increase, but on the other hand, similar removals during storage were observed without pH change and the confirmation of the affecting mechanisms (biological and chemical) require more research.

The study proposed that chemical (precipitation/sorption) of the studied pharmaceuticals was low or insignificant in all studied storage conditions, which was supported by the fact that, for example, in the groups of antibiotics and anti-tuberculotics in most assays the pH did not rise above 8, which is considered as a minimum pH prerequisite for the formation of struvite precipitates, with a pH-optimum of 9.4–9.7.[40] Previously, when the coprecipitation of CBZ and other pharmaceuticals (CBZ was the only same pharmaceutical as in the current study) with struvite in urine was investigated, it was proved using mass balances that the studied pharmaceuticals remained in the solution (> 96% of CBZ).[41] RMP was completely removed when pH was 9.4 (individually) as well as in pH 7.4 (urease inhibitor addition), suggesting that the precipitation with struvite was not the major removal mechanism. Adsorption on particulate matter, which is abundant in source-separated urine and was removed when samples were filtrated, was also regarded as a possibility. In practice, the role of adsorption could differ depending on adsorption sites in different conditions. Stability studies for the pharmaceuticals, for example, with purified water were chosen not to be conducted, since although they might have given some indication of the compound stability, ionic strength and other properties of human urine (see Table 2) are quite different from plain water, making comparison unequal.

Hypothesis was that the addition of faeces would supplement urine with additional bacteria thus improving compound removal. However, the results clearly showed that faeces did not enhance pharmaceutical removal even though they were expected to increase microbial content in assay jars. Faeces are practically always present in source-separated urine,[15] and it was hypothesized that the bacteria derived from faeces would enhance the biological removal of the compounds. Thus, the results imply that pharmaceutical removal may in fact be reduced in the presence of faecal contamination, but the mechanism is yet to be discovered.

It appeared that the used urease inhibitor resulted in a low pH increase as anticipated, but it did not, however, affect positively on pharmaceutical reduction. This indicates that the mechanism in pharmaceutical concentration reduction could well be biological and was inhibited by the urease inhibitor. Originally, the hypothesis was that lower pH generally enables better microorganism growth thus enhancing the biological removal of pharmaceuticals as bacterial extracellular enzymes can break down bonds in pharmaceutical molecules. In addition, the HPLC analysis showed that 59% of the urease inhibitor was still present in the sample after six months (data not shown). To our knowledge, no information is available regarding the urease inhibitor's effect on bacterial enzyme activity besides ureases. The addition of the urease inhibitor might have co-affected the removal of compounds by preventing such activity thus explaining the poor concentration reductions.

4. Conclusions

From the present study, we conclude that during the six-month storage of source-separated urine

- the pharmaceutical concentration reductions ranged from less than 1% to more than 99%: without amendments reductions were for anti-tuberculotics 41.9–99%; for antivirals <52% (except with 3TC 75.6%) and for antibiotic compounds <50%.
- in assays with amendments, the reductions were all <50% (except with RMP >99%). Transformation products were detected for 3TC, CIP and SMX, but the parent compounds were still present in urine after six months.
- RMP concentration reduced to below LOD in all assays and four unidentified transformation products were detected.
- faeces and urease inhibitor amendments resulted in similar or lower reduction than without them which was contradictory to the anticipation that the bacterial activity should increase and therefore improve concentrations reductions. The urease inhibitor prevented ureolysis and subsequent pH rise, but did not enhance pharmaceutical concentration reduction.
- biological activity was proposed by increased pH during storage, but similar removal during storage was observed without pH change. Thus, the

confirmation of the affecting mechanisms (biological and chemical) requires more research.

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No potential conflict of interest was reported by the authors.

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IV

USE OF DILUTED URINE FOR CULTIVATION OF *CHLORELLA VULGARIS*

by

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Use of diluted urine for cultivation of *Chlorella vulgaris*

Sanna Jaatinen, Aino-Maija Lakaniemi and Jukka Rintala

Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland

ABSTRACT

Our aim was to study the biomass growth of microalga *Chlorella vulgaris* using diluted human urine as a sole nutrient source. Batch cultivations (21 days) were conducted in five different urine dilutions (1:25–1:300), in 1:100-diluted urine as such and with added trace elements, and as a reference, in artificial growth medium. The highest biomass density was obtained in 1:100-diluted urine with and without additional trace elements (0.73 and 0.60 g L^{-1}, respectively). Similar biomass growth trends and densities were obtained with 1:25- and 1:300-diluted urine (0.52 vs. 0.48 gVSS L^{-1}) indicating that urine at dilution 1:25 can be used to cultivate microalgal based biomass. Interestingly, even 1:300-diluted urine contained sufficiently nutrients and trace elements to support biomass growth. Biomass production was similar despite pH-variation from < 5 to 9 in different incubations indicating robustness of the biomass growth. Ammonium formation did not inhibit overall biomass growth. At the beginning of cultivation, the majority of the biomass consisted of living algal cells, while towards the end, their share decreased and the estimated share of bacteria and cell debris increased.

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1. Introduction

Microalgae have been studied extensively for production as feedstock for bioenergy and biofuels (for reviews and discussion, see [1–4]). More sustainable cultivation of microalgal biomass can be achieved if the nutrients are obtained from recycled resources or waste materials.[5–7] Source separated urine is one possible waste-derived nutrient source for microalgae, as large amounts of nitrogen (25–35 g d^{-1} per person) and phosphorus (2–2.5 g d^{-1} per person) are excreted into human urine although N and P produced by an individual per day are highly dependent on the person and the diet.[8] The production rate of urine has been estimated to be 0.6–2.5 L $person^{-1}$ d^{-1}.[8] Decentralized sanitation concepts where wastewater is source separated and collected for use have gained more attention. They provide an alternative to recycle the nutrients in urine and can be considered to promote sustainable sanitation practices in some cases compared with centralized wastewater treatment systems.[9] The advantage of growing microalgae on urine is that they both treat urine by removing nitrogen and phosphorus from it and also produce high yield of biomass [10] which can be used in the production of, for example, biofuel applications.

The cultivation of microalgae in human urine has only recently started to gain more interest and not many studies are available that use real, non-sterilized urine. In algal cultivations with urine, the presence of bacteria (originated from urine) which might have a major impact on algal growth has neither been explicitly reported nor studied. In addition, to our knowledge microalga *Chlorella vulgaris*, which is considered a promising strain in biotechnology applications, has not been previously used in algal growth experiments with urine as a nutrient source. The growth of different microalgae in real and synthetic human urine has previously been studied using cyanobacterium *Spirulina platensis* [11,12] and microalgae *Chlorella sorokiniana* [13,14] and *Scenedesmus acuminatus*.[15] For example, Tuantet et al. [14] grew *C. sorokiniana* in 1:2–1:3-diluted human urine to produce biomass and obtained a high biomass productivity of 9.3 g L^{-1} d^{-1} (per reactor volume) with a continuous reactor design. All of the studies have demonstrated microalgae's ability to use nutrients (nitrogen and phosphorous) from urine. Interestingly, when using real human urine, cultivations have been inoculated with algae and supplemented with non-sterilized urine which most likely leads to bacterial contamination of the cultivations. A large variety of bacteria have been detected from *C. vulgaris* cultures grown on both sterilized and non-sterilized artificial growth media,[16,17] emphasizing the fact that bacteria have likely been a significant part of algal cultures grown on urine.

Production of nitrogen fertilizer has been noted as an important financial and a sustainability factor in microalgal cultivations and its effect on chemical biomass

CONTACT Sanna Jaatinen sanna.jaatinen@tut.fi

composition has been investigated in many publications. Different nitrogen sources (ammonium, nitrate and urea) have been studied for cultivating different microalgae. [10,18] Most of the nitrogen in urine is originally in the form of urea (~85% [8]), which however, during storage of urine, is hydrolysed resulting in the release of ammonium and ammonia. Hulatt et al. [18] showed that cultivation of *Chlorella vulgaris* with medium containing urea as the nitrogen source had a slightly higher maximum biomass growth rate and yield than with nitrate, while no growth occurred with ammonium. The reason for latter was that ammonium utilization caused a significant decrease in culture pH (from 6.8 to 4) and resulted in the cessation of *C. vulgaris* growth. However, according to tests conducted by Schuler et al.,[19] *C. vulgaris* prefers ammonium over nitrate as the nitrogen source suggesting that urine would make a preferable nitrogen source in algal cultivations. In previous studies,[10,11,14,15] microalgal growth in diluted human urine has been studied successfully using additional trace elements to enhance growth, which has raised a research need regarding growth without trace element supplementation to produce algal biomass more sustainably and cost-efficiently.

To promote further studies on the realization of microalgal cultivation in source separated urine, at least two types of scenarios for full-scale systems can be speculated. The first scenario would entail quite concentrated urine diluted with water [13,14] with high light intensities (>1000 µmol photons m^{-2} s^{-1}) and a continuous reactor design with CO_2 and trace elements additions to sustain growth. In the second scenario more diluted urine (e.g. dilution 1:25) would be used [10,11,15] in batch mode without CO_2 and trace element addition, and at low illumination. It can be speculated that the second scenario would be more economical as in the first scenario the additions would increase costs, energy requirements and environmental impacts. On the other hand, these impacts could be compensated at least partly by the increased biomass productivity. Thus, case-related economic and life cycle analyses (LCA) should be used to enable more conclusive comparison. To our knowledge, LCA of cultivation of microalgae on source separated (hydrolysed) urine is only shortly discussed while LCAs on algae cultivation on wastewaters has recently been reported [20,21]; thus, an economic comparison of the two scenarios is not possible at the moment. In practice, several factors affect the realization and localization of these kind of systems such as the scale of the system (urine volume, etc.), availability of light, dilution water and CO_2,[22] and required reactor structures as well availability of land. Furthermore, algal cultivation is only part of the system and both processes would require biomass harvesting, processing and utilization as well as final treatment of the diluted urine.

Based on the second scenario presented above, the aim of this study was to evaluate the biomass growth of microalga *C. vulgaris* using diluted human urine as a sole nutrient source. The hypothesis was that *C. vulgaris* can grow in diluted human urine without trace element additions, and thus the trace element addition, CO_2 supplementation and high degree of culture optimization could be avoided. Urine used in the experiments was not autoclaved and thus contained bacteria. No CO_2 addition was used and the illumination was kept low to study the growth in less optimized conditions. First, *C. vulgaris* was cultivated in 1:100-diluted urine as such and with trace elements and as reference in artificial growth medium with nitrate as the nitrogen source. Subsequently, the effect of urine dilution on algal growth was studied. The experiments included the analysis of total biomass as dry weight (algae and bacteria) and algal cell count to reveal the share of living algae of the total biomass (referred to as microbiological composition). To our knowledge, the microbiological composition of biomass grown in human urine has not been previously studied. Yet, it is of particular interest regarding biomass utilization options, for example, anaerobic digestion.

2. Materials and methods

2.1. *Microalga, maintenance and urine*

C. vulgaris (SAG 211-11b) was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen. Maintenance cultures and two inoculum cultures of *C. vulgaris* were grown in modified Chu-10 medium,[23] where Na_2SiO_3, H_2SeO_3, Na_2EDTA and citric acid were left out as they were not required for growth of *C. vulgaris*. Chu-10 medium was chosen as the reference medium (to compare algal growth in urine vs. artificial medium) since Ilavarasi et al. [24] compared five different typical microalgal growth media for the cultivation of *C. vulgaris* (Modified Hoagland's medium, Bold's Basal medium, Acidified Bold's Basal medium, Half strength Chu-10 medium and BG 11 medium) and reported the highest biomass production using half strength Chu-10.

The modified Chu-10 medium for the maintenance cultures and inoculum growth was prepared in MilliQ-water and contained (mg L^{-1}): $Ca(NO_3)_2 \times 4H_2O$ (57.56), K_2HPO_4 (10.0), $MgSO_4 \times 7H_2O$ (25.0), Na_2CO_3 (20.0), $FeCl_3 \times 6H_2O$ (1.334), H_3BO_3 (2.86), $MnCl_3 \times 4H_2O$ (1.81), $ZnSO_4 \times 7H_2O$ (0.222), $CuSO_4 \times 5H_2O$ (0.79), $Na_{26}MoO_4 \times 2H_2O$ (0.39), $Co(NO_3)_2 \times 6H_2O$ (0.0494) and vitamins – thiamin B_1 (0.2), biotin B_7 (0.001) and cyanocobalamin B_{12} (0.001).

The total concentrations of N and P in the modified Chu-10 medium were 0.49 and 0.06 mM, respectively. The pH of the medium was set to 6.4 with 1 M HCl and the medium was sterilized by autoclaving. Maintenance cultures were transferred into fresh sterile medium at four-week intervals to maintain the culture. Maintenance cultures were grown in 250 mL Erlenmeyer shake flasks at 135 rpm mixing, 24 ± 1.0°C and under a constant illumination of 29 µmol photons m^{-2} s^{-1} (5 fluorescent OSRAM L 18W/965 Biolux-lamps, light intensity measured with a DeltaOHM HD 9221 lux meter). This light intensity is relatively low, but light intensities of similar magnitude (39–70 µmol photons m^{-2} s^{-1}) have successfully been used for microalgal cultivation.[15,25,26] No air or CO_2 was added to the cultivations by sparging. The only source of carbon was the surrounding air, the introduction of which to the cultures was enhanced by shaking of the cultures. No CO_2 addition was used since our previous findings have demonstrated that the culture pH with the fresh water strain *C. vulgaris* is markedly lower when CO_2-enriched air is fed to the cultures, unless the culture medium is well buffered.[17]

Two inoculum cultures (to grow sufficient amount of inoculum) of *C. vulgaris* were grown in 500 mL of modified Chu-10 medium in similar conditions as the maintenance cultures. The urine used in the experiments was collected from a healthy male volunteer receiving no medication. Two different batches of urine were used, one for each experimental set-up (Table 1).

2.2. Growth experiment set-ups

Two experimental set-ups were used: in set-up I different growth media were compared and in set-up II different urine dilutions were studied. Both growth experiments were conducted in 1 L Erlenmeyer flasks for 21 days in similar conditions (mixing, temperature and light intensity) as the cultures used as inoculum. Experiments were conducted as triplicate (set-up I) and duplicate (set-up II), as is typically done in microalgal studies employing batch cultivations.[12,15] The temperature was 25.5 ± 1.3°C during set-up I and the dissolved oxygen (DO) varied between 7.9 and 9.5 mg L^{-1} (saturation 96.7–106.9%). The temperature in set-up II was 24.2 ± 1.4°C and DO varied between 7.9 and 9.3 mg L^{-1} (saturation 95.6–109%). To inoculate the experimental cultures, an aliquot (60 mL) was harvested during the exponential growth phase from the inoculum cultures into 600 mL of selected experimental growth medium.

In set-up I, *C. vulgaris* was grown in three media (*i–iii)* having the concentrations of N_{tot} and P_{tot} 5.0 mM (70.6 mg L^{-1}) and 0.2 mM (6.4 mg L^{-1}), respectively (N:P ratio of 25:1). The growth media were (*i*) 1:100-diluted urine (C:N 0.15), and as reference (*ii*) 1:100-diluted urine with trace elements from Chu-10 medium (referred to as 'urine+TE', C:N 0.75) and (*iii*) modified Chu-10 medium, where the concentrations of N_{tot} and P_{tot} were adjusted to match that of 1:100-diluted urine (an additional amendment of $Ca(NO_3)_2 \times 4H_2O$ and K_2HPO_4 to obtain the N_{tot} and P_{tot} concentrations corresponding to the levels of 1:100-diluted urine, C:N 0.83). This adjustment was done to ensure a similar concentration of N and P in all cultures. The pH of all three media preparations was adjusted to pH 6.4 with 1 M HCl after which the media were autoclaved without urine present, after which urine was added in relevant shake flasks.

In set-up II, the effect of urine dilution was investigated. *C. vulgaris* was grown for 21 days in urine dilutions of 1:25 (C:N 0.74), 1:75 (C:N 0.76), 1:100 (C:N 0.88), 1:150 (C:N 1.2) and 1:300 (C:N 1.3). The pH of MilliQ-water used for diluting the urine was adjusted to pH 6.4 with 1 M HCl to correspond the pH-adjustment in set-up I before autoclaving. Urine was added after autoclaving of the MilliQ-water into each flask to obtain relevant dilution.

Table 1. The characteristics of undiluted urine used in the two experimental set-ups.

Urine composition (as mg L^{-1} where appropriate)	Set-up I	Set-up II
pH	5.8	5.7
N_{tot}	6800	4320
NO_3^-–N	<5	<2
NO_2^--N	<1	<1
NH_4^+-N	520	275
N_{org}	6275	4042
P_{tot}	670	355
DOC	4830	2405
Fe[a]	<1	n.a.
Mg	50	n.a.
Ca	77	n.a.
Na	710	n.a.
K	717	n.a.

Note: n.a. = not available.
[a]Below AAS detection limit.

2.3. Analyses and calculations

DO was measured using a WTW Multiline P3 meter with a WTW CellOx 325 electrode and pH was measured using a WTW 3210 pH-meter with a SenTix 41 pH-electrode. Optical density (OD) was determined by measuring absorbance at 600 nm using a Shimadzu UV-1700 PharmaSpec spectrophotometer. OD measurement was used to calculate the growth rate since it required only small sample volume. If necessary, the samples were diluted with MilliQ-water to give OD below 1. Growth rate was calculated as $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 is the OD at time t_1 and N_2 is OD at t_2. Calculations were based on the following: In set-up I, OD increased

from 0.099 to 0.640 (days 0–5) in modified Chu-10; from 0.074 to 1.15 (days 0–9) in urine+TE; from 0.067 to 0.762 (days 0–9) in 1:100-diluted urine. In set-up II, OD increased from 0.086 to 1.26 (days 0–7) in 1:25; from 0.085 to 0.93 (days 0–7) in 1:75; from 0.53 to 1.46 (days 11–16) in 1:100; from 0.086 to 0.408 (days 0–7) in 1:150; from 0.081 to 1.18 (days 0–7) in 1:300.

Volatile suspended solids (VSS) were determined by filtering a measured volume of culture solution through a glass fibre filter (Whatman GF/A), followed by drying the filter at 105°C for 20 h and igniting it at 550°C for 2 h. Assumption was that VSS measures the algal biomass and the dead algal cells and bacteria present in the cultures. Algae cell counting (including only intact algal cells) was performed with phase contrast microscopy (Zeiss Axioskop 2) using a Thoma counting chamber (0.02 mm depth, Fischer Scientific). However, a slight uncertainty to algal biomass calculations comes from the fact that at the midpoint of the cultivation (around days 7–11), in all dilutions but most visibly in 1:100-diluted urine (in set-up II), the algal cells formed larger clumps, that could not be enumerated in cell counting thus decreasing the calculated cell concentration. This is not, however, believed to have a significant effect on the share of algae in the cultivations (max. 10% by rough estimation). The algal cell count was converted to VSS_{algae} by multiplying average algal cell count with an average *C. vulgaris* cell mass of 25.7 pg [27] to estimate which part of the total biomass (VSS) was composed of algae. By using this value in calculations, it was confirmed that the algal cell weight was not underestimated, as other cell masses found in the literature for *C. vulgaris* in different growth phases and experimental set-ups were lower than the average reported by de-Bashan et al.[27] The fraction of bacterial biomass and dead algal cells was calculated as VSS-VSS_{algae}. The method for calculating VSS_{algae} was the same as previously used by Perez-Garcia et al. [25] to estimate dry weight of *C. vulgaris* in batch cultivations. Measurements of proteins, lipids and hydrocarbons from the produced biomass were out of scope of this study. Previous study [18] has demonstrated that lipid and protein content at different stages of *C. vulgaris*' growth are not markedly affected by the nitrogen source, which is why the effect of urea as the nitrogen source on cell composition was not examined further.

Ion chromatography (IC, Dionex ICS-1600) was used to determine the anions of interest (PO_4^{3-}, NO_3^-, NO_2^-) from the filtered (0.45 µm nylon syringe filters, VWR) culture samples. The separating column applied was an IonPac AS4A-SC anion exchange column with ASRS-300 suppressor (2 mm). The eluent used was a buffer containing 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$. Analysis of the samples was done according to the ion chromatography standard SFS-EN ISO 10304–1:en.[28]

Fe, Na, K, Mg and Ca were analysed from urine with an atomic adsorption spectrophotometer (AAS) AA200 analyser (Perkin Elmer) and air acetylene flame. The filtered samples were diluted at ratios ranging from 1:10 to 1:1000 with MilliQ-water using acid washed glassware. A 5% (v/v) CsCl–LaCl solution (10 g L^{-1} CsCl + 100 g L^{-1} La, Merck) was added to the samples of which Ca was analysed to stop the formation of substances which inhibit Ca atomization. Fe analysis was performed from samples diluted in 0.07 M HNO_3 to prevent iron oxidation. The analysis was done according to the SFS standards 3018 [29] and 3044 [30].

Dissolved organic carbon (DOC) was determined using a Shimadzu TOC-5000 Analyzer according to the Finnish standard SFS-EN 1484:en [31] from filtered samples. Total phosphorus (P_{tot}), ammonium nitrogen (NH_4^+-N) and total nitrogen (N_{tot}) were analysed from filtered samples using HACH Lange cuvette tests and HACH spectrophotometer DR 2800. The organic nitrogen (N_{org}) in the samples was calculated by subtracting the concentrations of NO_3^--N, NO_2^--N and NH_4^+-N from N_{tot}. The C:N ratios at the beginning of the cultivations (day 0) were calculated from the DOC and N_{tot} concentrations to provide insight on the culture conditions. The C:N ratio has been shown to have a significant effect on lipid production at least with heterotrophic microorganisms.

3. Results and discussion

3.1. *Biomass growth*

Growth of biomass in *C. vulgaris*-inoculated cultivations were studied in 1:100-diluted urine and as a reference in 1:100-diluted urine with additional trace elements from Chu-10 medium and in Chu-10 medium with artificial N and P (Figure 1(a)) as well as in different urine dilutions (1:25, 1:75, 1:100, 1:150 and 1:300, Figure 1(b) and 1(c)).

The biomass growth, measured as VSS thus including both living and dead algae and bacteria at least in cultivations containing urine (in both set-up I and II), followed similar growth rates and final biomass concentrations (0.5–0.6 gVSS L^{-1}, Table 2) for all urine dilutions as well as for all reference cultures. Somewhat higher VSS concentrations were obtained with 1:100-dilution (Figure 1(b)) and urine+TE (Figure 1(a)), and slightly lower with 1:300-diluted urine (Figure 1(c)).

Based on algal counts (set-up II), which in maximum ranged from 8.0×10^6 to 6.0×10^6 cells mL^{-1} (dilutions 1:25–1:300), living algae dominated the biomass for the first 5–15 days in the incubations contributing about

50–100% of the biomass (in 1:100-dilution <30%, Figure 1(b)) after which the concentration of living algal cells stabilized while total VSS continued to increase. At the end of the incubations (day 21), the estimated portion of microalgae was less than 40% of the VSS in all dilutions (Figure 1(b) and 1(c)). However, it should be taken into account that this algal dry weight is only an estimate. It is acknowledged that the algal cell weight varies depending on the growth phase.[32]

The high share of algal VSS in the beginning of the cultivation (Figure 1(b) and 1(c)) suggests that in the beginning the conditions at all urine dilutions favoured microalgal growth while in the course of incubation, bacteria became more competitive and also the portion of dead algal cells increased. The trend was clear even though the share of algae in the biomass may be somewhat underestimated due to clump formation which complicated cell counting. This is not believed to have a significant effect on the estimated share of algae in the cultivations. Clump formation in algal cultivations in the presence of bacteria and their extracellular polymeric substances has been previously reported.[33] In addition, the intracellular substances released by dead algae might have affected clump formation. These are likely reasons for clumping observed in the present study, since clump formation coincided with the beginning of the stationary phase in algal growth (Figure 1(b) and 1(c)) and increasing share of bacteria in the biomass. The growth in 1:100-diluted urine was compared with other dilutions unexpectedly poor in both replicates in set-up II, which cannot be explained.

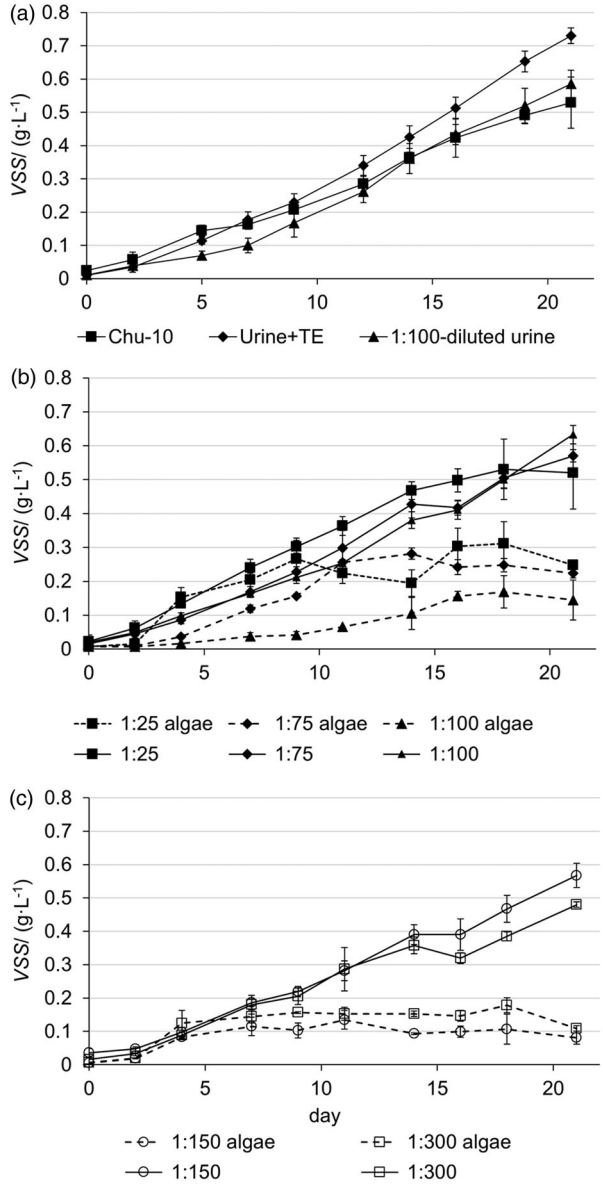

Figure 1. Biomass production as VSS in (a) set-up I using 1:100-diluted urine, urine+TE and Chu-10, and as VSS and calculated VSS_{algae} (b) in set-up II with urine dilutions 1:25, 1:75 and 1:100, and (c) urine dilutions 1:150 and 1:300. The error bars represent the standard error of three (a) and two (b, c) replicates.

The percentage of living algae of the produced biomass was larger in less diluted urine (1:25 and 1:75, Figure 1(b)) although it could be assumed that in that case also the amount of added bacteria was the largest. The use of less diluted urine seems to be the most beneficial to microalgal growth since the nutrients are the most abundant and the algal biomass growth rate is the highest. This deduction is also supported by results of Tuantet et al.[14] who concentrated on the optimization of *C. sorokiniana* cultivation in photobioreactors with a very short light path using, for example, trace mineral additions.

As the microalgal and bacterial growth (VSS) was almost similar in all urine dilutions (Figure 1(a) and 1(c)), and VSS was similar when comparing 1:100-diluted urine with Chu-10 medium, it seems that all cultivation media sufficiently contained elements for the growth of algae and/or bacteria. For example, the 1:300 and 1:25-dilutions (Figure 1(b) and 1(c)) resulted in similar final VSS concentrations (0.48 vs. 0.52 gVSS L^{-1}, Table 2) suggesting that the used culture conditions were possibly growth limited either by light or by CO_2 rather than nutrients. After the initial growth of algae, their further growth was likely constrained by the limited CO_2 availability or light transfer (29 µmol photons m^{-2} s^{-1}) due to increasing biomass concentration (microalgal VSS remained a little lower than 0.3 gVSS L^{-1} even at the lowest urine dilution, Figure 1(c)) or different kinetics of algae and bacteria. Apparently the dead algal cells, which were also observed through microscopical observations ('empty shells'), released substrate/carbon for the growth of bacteria in the course of the incubations and are also present in the biomass portion not consisting of living algal cells.

Table 2. Results from *C. vulgaris* growth experiments.

	Reduction percentage (%)						
	N_{tot}	P_{tot}	NH_4^+-N[a]	N_{org}[a]	μ (d^{-1})	Biomass (gVSS L^{-1})[b]	Biomass productivity (gVSS L^{-1} d^{-1})[b]
Chu-10	33	>99	−128	−11	0.37	0.53 (0.02)	0.06 (0.02)
urine+TE	45	>99	92	45	0.31	0.73 (0.08)	0.08 (0.03)
1:100-diluted urine	57	94	−86	70	0.27	0.59 (0.04)	0.06 (0.03)
1:25	40	35	−366	71	0.38	0.52 (0.11)	0.06 (0.03)
1:75	55	54	−166	80	0.34	0.57 (0.02)	0.07 (0.03)
1:100	74	80	−70	84	0.20	0.60 (0.03)	0.07 (0.04)
1:150	77	84	25	80	0.35	0.57 (0.04)	0.06 (0.03)
1:300	79	92	42	81	0.38	0.48 (0.01)	0.05 (0.03)

Note: The reduction percentage of the main nutrients describes to what extent it was used. μ is the specific growth rate of the algae, biomass describes the biomass concentration in the end of the experiment and the average biomass production (calculated from days 0 to 21) represents the biomass produced on a daily basis.
[a]Negative value means that nutrient was released from the microorganisms.
[b]Mean (standard error).

In previous studies on the cultivation of microalgae in human urine,[10,11,15] the microbiological composition in the biomass has not been investigated (Table 3); cultivations were inoculated with microalgae and it was assumed that the obtained biomass consisted of algae only. In the current study, the colour of the cultivations was dark green typical for algae cultivations; however, it did not ensure that only algae were present. Biomass growth in previously mentioned studies has been measured with OD and dry weight (biomass filtered and dried overnight at 105°C), which, to our knowledge measure, both living and dead algae and bacteria. In the previous study performed by Tuantet et al. [14] on algal growth in urine, the observed 71% COD removal was reasoned to be due to bacterial presence, while the concentration of bacteria was not estimated, for example, by microscopy examination, cell counting or molecular biology methods. However, *Chlorella* is capable in growing heterotrophically by using DOC,[34] but the presence of dissolved organic substrates likely leads to increased competition with other microorganisms, for example, bacteria,[25] which is probably one of the reasons for low algal biomass in this study.

The source for bacteria in cultivations is obvious as urine contains bacteria, whose presence was microscopically confirmed in this study. The presence of bacteria in the urine containing growth medium was not prevented, for example, by autoclaving since it was found that autoclaving enhanced the urea transformation to ammonium thus significantly changing the urine composition. In addition, it was deemed that sterilization of urine would not be possible in a real scale process utilizing urine due to the high costs and energy requirement of autoclaving. The avoidance of bacterial growth, in fact, is not needed if the produced biomass is considered for methane production, as previous research has shown that the biomass consisting of bacteria and microalgae can give even higher methane yields than pure microalgal biomass. For example, Lü et al. [35] showed that in batch system 17–24% higher methane yields (376 to 403 mL $CH_4 \cdot gVS^{-1}$) were obtained from

Table 3. Comparison of culture conditions and biomass production in this study and other microalgal cultivation studies conducted using human urine.

Alga	Substrate	Cultivation conditions	Biomass (g L^{-1})	Biomass growth measured	Microorganism composition determined	Reference
C. sorokiniana	1:3-Diluted urine	Continuous flat plate reactor, short light path (10 mm) CO_2, Mg, Fe supplementation, antifoaming agent addition, N and P optimization, 1550 μmol photons m^{-2} s^{-1}	15.4	Optical density	no	[14]
S. platensis	1:120-Diluted urine	1.2 L Photobioreactor (batch), CO_2 supplementation, 2000 μmol photons m^{-2} s^{-1} [a]	0.81	Optical density	no	[10]
S. platensis	1:180-Diluted urine	1.2 L photobioreactor (batch), CO_2 supplementation, 2000 μmol photons m^{-2} s^{-1} [a]	2.32	Dry weight chlorophyll	no	[11]
S. acuminatus	1:50-Diluted urine	Batch bottles (200 mL), Mg, Fe supplementation, 39–47 μmol photons m^{-2} s^{-1}	0.16[b]	Optical density dry weight	no	[15]
C. vulgaris	1:25–1:300-Diluted urine	Batch bottles (600 mL), 29 μmol photons m^{-2} s^{-1}	0.60	Optical density dry weight Microscopical cell enumeration	Yes	This study

[a]444.4 W m^{-2} converted to μmol photons m^{-2} s^{-1} using a conversion factor of 1 W m^{-2} = 4.5 μmol photons m^{-2} s^{-1}.
[b]Calculated. The author converted dry weight to million cells mL^{-1} (115 mg dry weight·L^{-1} = 1 × 10^6 cells mL^{-1}).

biomass containing both algae (*C. vulgaris*) and bacteria (1–10%) than biomass containing only algae.

In this study, the obtained biomass production rates (0.05–0.07 g L^{-1} d^{-1}, μ 0.20–0.38 d^{-1}) and final biomass concentrations 0.48–0.60 gVSS L^{-1} (diluted urine, Table 2) were in line with other algal growth experiments reported in the literature, although not especially high. Lam and Lee [26] obtained 0.29–0.51 g L^{-1} biomass and μ of 0.23–0.27 d^{-1} in a 12-day cultivation period using organic fertilizer medium, continuous aeration with compressed air and light intensity of 60–70 µmol photons m^{-2} s^{-1}. A biomass production rate of 0.05 g L^{-1} d^{-1} and maximum biomass concentration of 0.53 g L^{-1} were obtained when *C. vulgaris* was grown on urea in a column photobioreactor at a light intensity of 225 µmol photons m^{-2} s^{-1}.[18] However, in that study, the concentration of nitrogen (urea) was kept low (1.33 mM) to induce lipid accumulation inside the cells and this can have slowed down the growth. Higher biomass concentrations (15.4 g L^{-1}) have been obtained on urine supplemented with trace elements using high light intensity (490–1550 µmol photons m^{-2} s^{-1}) in laboratory scale (~1 L) with a continuous reactor design (Table 3, [14]). However, the design of the reactor and process conditions, including the addition of trace elements, differed from the present experimental design, making the comparison of biomass production and scalability of the systems inequitable.

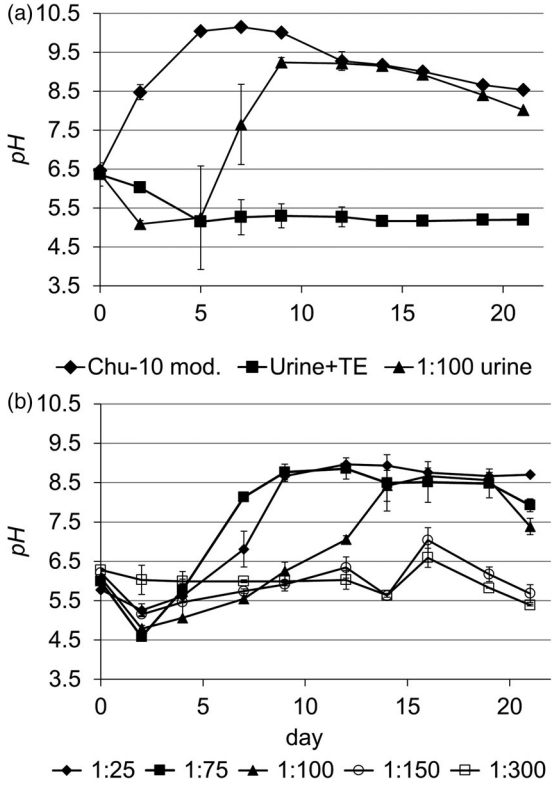

Figure 2. Cultivation pH: (a) set-up I with 1:100-diluted urine, urine+TE and Chu-10 medium, (b) set-up II with different urine dilutions. The error bars represent the standard error of (a) three and (b) two replicates.

Availability of trace elements is considered a potential limiting factor when cultivating algae with urine. The addition of trace elements such as iron and magnesium was shown to improve biomass production in human urine.[13,14] The present studied urine+TE medium also contained added vitamins, magnesium and iron. Undiluted urine contains more magnesium and approximately the same concentration of total iron as Chu-10 (Table 1). Dilution of urine and thus decreased trace element concentrations did not affect overall biomass growth in this study. The lower concentration of trace elements may have led to bacterial dominance in the cultures because there may not have been enough magnesium or iron to support the growth of photosynthetic algae, which require these elements, for example, for the production of chlorophyll and photosynthetic enzymes. However, when comparing biomass growth in 1:100-diluted urine without additional vitamins and trace elements (set-up I) with modified Chu-10 medium, it was demonstrated that urine without any additional vitamins or trace elements can sustain microalgal growth.

3.2. *pH and DOC*

In all cultures, the starting pH was 6.2–6.6 (Figure 2(a) and 2(b)) from which in urine dilutions 1:25–1:100 the pH increased after an initial drop (5–6) up to 8–10 within 5–10 days while some decrease towards the end of the incubation was seen (Figure 2(b)). Contrary to those, pH remained lower (5–6) during the whole incubation time with higher urine dilutions (1:150–1:300, Figure 2(b)) and with urine+TE (Figure 2(a)). The fact that biomass growth was similar during the cultivations even though pH varied greatly between different cultivation batches indicates the robustness of the biomass production since the growth was not limited by the culture pH. The decreased pH at the beginning of cultivations with urine (Figure 2(a) and 2(b)) was apparently due to ammonium uptake by algae, which results in H^+ production lowering pH.[36] This is supported by the observation that no such decrease was seen in Chu-10 medium since nitrogen was added as nitrate and in higher dilutions, in which the starting concentration of ammonium was quite low. The increased pH in the cultivations after a few days was likely due to algal assimilation of HCO_3^- via photosynthesis.

Plummeting pH values have been detected in the batch cultivation of *C. vulgaris* grown with ammonium

in a photobioreactor where low pH (drop from pH 6.8 to less than 4) resulted in the cessation of algal growth,[18] while the growth-limiting pH for *C. vulgaris* has been reported to be 3.5–4.5.[37] Urea uptake by algae should not affect the pH.[36] Some bacteria that inhabit the urinary tract produce ureolytic enzymes [38] which during the storage of urine increase the pH up to 9 leading to urea transformation into ammonium. Since urine amended was not sterile, the ureolytic activity of bacteria present in the urine has likely caused the release of ammonium which may also have increased the cultivation pHs.

The initial DOC (Figure 3(a) and 3(b)), which originated from the inoculum and the urine, ranged between 8.4 and 114 mg L^{-1} and was the highest with the highest urine concentration. DOC in 1:25-diluted urine decreased throughout the experiment, while the DOC in the other four dilutions began to rise after 10 days. The DOC in 1:100-diluted urine rose 60%, whereas in the urine+TE it remained unchanged (Figure 3(a)). After the microalgal growth reached its peak based on algal cell counts, DOC seemed to follow VSS in cultures with urine diluted 1:75 or more (Figure 1(b) and 1(c)). This was probably due to the decay of algal cells, which was followed by enhanced bacterial growth. In 1:25-diluted urine, the concentration of DOC originated from urine (Figure 3(b)) was so high that the DOC released by *C. vulgaris* could not be detected.

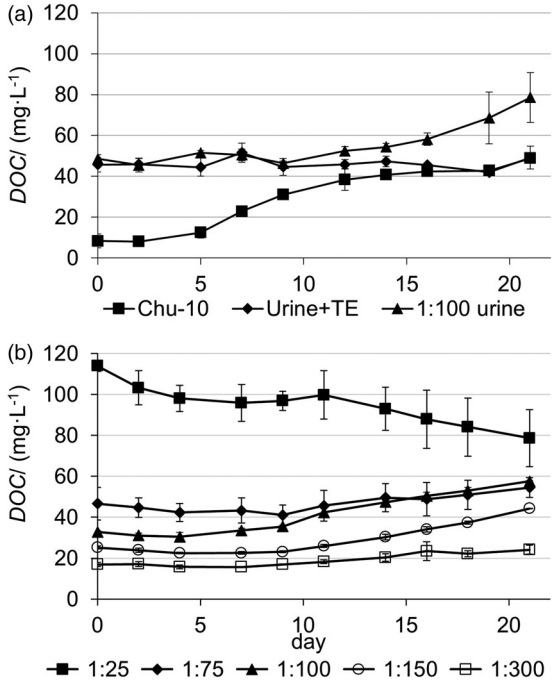

Figure 3. Dissolved organic carbon (DOC) in set-up I (a) and set-up II (b) at different urine dilutions. The error bars represent the standard error of three (a) and two (b) replicates.

3.3. *Nutrient effects*

Dissolved P_{tot} concentration (Figure 4(a) and 4(b)) decreased in 5–11 days below 50 µg (detection limit) in modified Chu-10 medium and in urine+TE and in 1:300-diluted urine while with less diluted urine, P_{tot} also decreased until day 11, but after that remained unchanged. In 1:25-diluted urine final P_{tot} concentration was 9.4 mg L^{-1} (Table 2, Figure 4(b)). The different evolution of P_{tot} for the two 1:100-diluted urine in the two set-ups (Figure 4(a) and 4(b)) is likely due to the differences in the urine as in set-up I, for example, phosphorus concentration was almost double to that of set-up II (Table 1). Based on the algal cell counts, decreasing P_{tot} concentration in the cultivations followed the algal growth (Figure 4(b)). When the algae cell counts reached a stationary phase, the P_{tot} consumption also slowed down. Thus, the algae might have utilized phosphorus in their growth in the stationary phase and the dead algae might have released phosphorus for the bacteria to utilize (Figure 4(b),

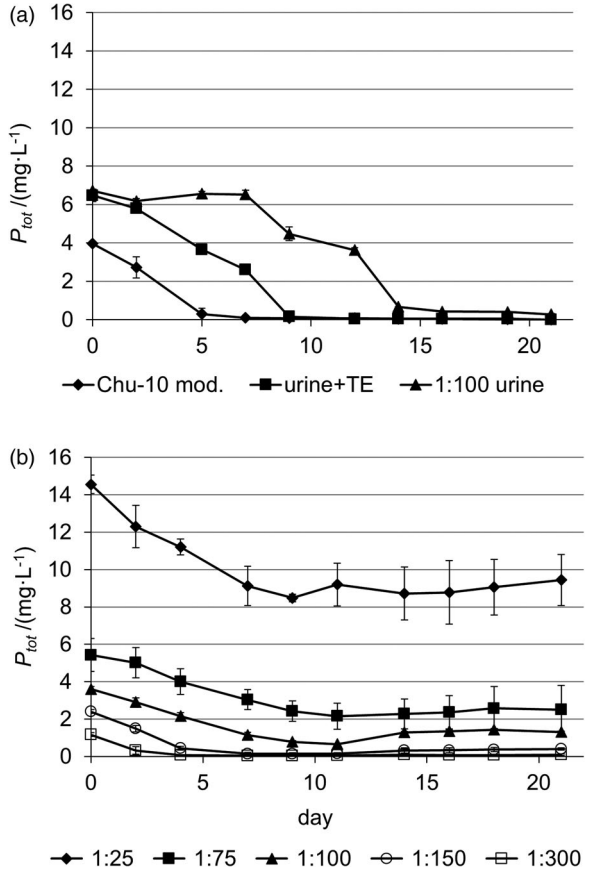

Figure 4. Phosphorus utilization (P_{tot}). (a) set-up I with 1:100-diluted urine, urine+TE and Chu-10 medium, (b) set-up II with different urine dilutions. The error bars represent the standard error of (a) three and (b) two replicates.

around day 12). It appears that P_{tot} did not limit the overall biomass production as VSS continued to increase (Figure 1(b) and 1(c)). Most microalgae can grow even at as low concentrations as 50 μg L^{-1} phosphorus.[39] However, in the present study, overall biomass was not composed of algae alone.

In cultivations with urine, N_{tot} (dissolved, Figure 5(a) and 5(d)) was initially mainly N_{org} while with Chu-10 medium nitrogen was added as nitrate (Figure 5(b)). During the cultivations, N_{tot} concentration (and calculated N_{org} by subtracting other nitrogen compounds from N_{tot}) reduced in all dilutions likely due to algal assimilation of urea (and/or bacterial metabolism). However, after 5–10 days of incubation, ammonium concentrations (Figure 5(c) and 5(f)) began to rise suggesting that bacteria, which started to dominate the cultures as discussed above, began to transform urea to ammonium.

The $NH_4^+ - N$ (Figure 5(f)) concentration increased in all dilutions rising up to 19 mg L^{-1} (1:75-diluted urine) but after 16–18 days the concentration started to level off except in 1:25-diluted urine, where the ammonium transformation continued due to high initial urea concentration (>50 mg L^{-1} $NH_4^+ - N$ by day 21, Figure 5(f)). In cultures to which urine was added, the urea concentration (N_{org}) decreased in all cultivations when the

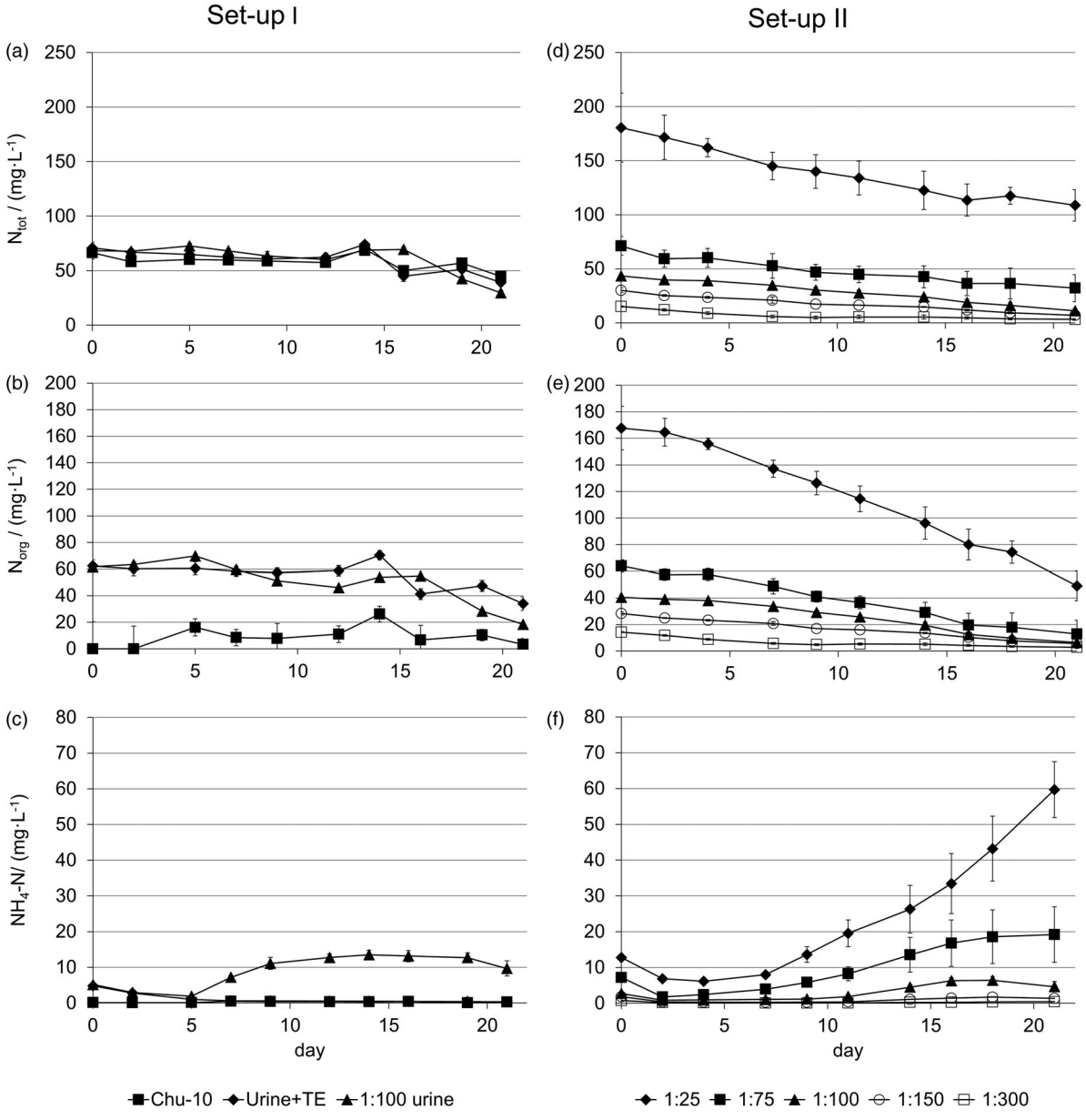

Figure 5. Nitrogen utilization as N_{tot}, N_{org} and NH_4–N in set-up I (a, b, and c) and with different dilutions in set-up II (d, e, and f). The error bars represent the standard error of three (set-up I) and two (set-up II) replicates.

concentration of ammonium started to increase, which was as expected as bacteria released ammonium from N_{org} in addition to algae and bacteria utilizing N_{org} in their growth. In the modified Chu-10 culture, nitrate (NO_3^-) was used as the nitrogen source and was converted into nitrite (NO_2^-) (93% decrease in nitrate concentration), the concentration of which increased up to level of 28 mg L^{-1} by the end of the incubation (data not shown). The concentration of nitrite remained below 5 mg L^{-1} in 1:100-diluted urine and in urine+TE cultures in set-up I and in set-up II nitrate was not detected during cultivation suggesting that nitrification did not take place. The absence of nitrite in algal cultivations on urine indicated that nitrifying bacteria were likely not introduced in the cultures via urine to convert urea to nitrite, whereas in modified Chu-10 culture nitrite was the logical product of nitrate (nutrient source) utilization.

The concentration of dissolved N_{tot} followed with the decline in N_{org} which indicated that the algae and bacteria utilized N_{org} (Figure 5(b) and 5(e)). According to Hodson and Thompson,[40] *C. vulgaris* converts urea to ammonium without enzymatic reactions. This suggests that *C. vulgaris* does not have ureolytic enzymes and indicates that in the present study *C. vulgaris* utilized urea as the nitrogen source, but ammonium formation was likely of bacterial origin. As stated previously, the ureolytic activity of bacteria found in urine can also have caused the decrease of N_{org} in the cultures. Since VSS_{algae} reached a stationary phase after around 10 days (Figure 1(b) and 1(c)), it is reasonable to assume that part of the urea consumption after that is due to bacterial metabolism.

Especially, the effects of ammonium containing urine as the nitrogen source on the growth of algal cultures were of interest as previously it has been shown that ammonium uptake lowers the culture pH thus affecting algal growth. Furthermore, unionized ammonia (as NH_3) can be toxic to microalgae [41] as shown by Adamsson [15] who reported decreased microalgal growth as ammonium concentration increased (2% urine was used). However, in the present study, the biomass concentration showed no signs of decline suggesting that overall biomass (including bacteria) growth continued despite the increasing ammonium concentration (after day 10, Figure 5(c) and 5(f)). The utilization of ammonium was different in the two set-ups: whereas in set-up I the concentration of ammonium rose rapidly after day 5, in set-up II it only began to rise after day 11 (Figure 5(c) and 5(f)). This was likely due to different initial ammonium concentrations in the two experiments, as the initial concentration of $NH_4^+ - N$ in set-up II was about half of that in set-up I (Table 1).

4. Conclusions

From the present study, we conclude that in a batch system using non-sterilized urine

- At the relatively low light intensities used, similar biomass yields of *C. vulgaris* and associated bacteria were obtained with 1:25- and 1:300-diluted urine (0.52 vs. 0.48 gVSS L^{-1}) as in artificial media previously found optimal for *C. vulgaris* cultivation.
- The highest biomass yields were obtained in 1:100-diluted urine with (0.73 gVSS L^{-1}) and without trace element additions (0.60 gVSS L^{-1}).
- Even 1:300-diluted urine contained sufficiently nutrients and trace elements to support biomass growth.
- The cultures were first dominated by living microalgae, while in the course of incubation bacteria became more competitive and also the portion of dead algal cells increased.
- Biomass growth was similar in comparative cultivations although pH varied from <5 to 9.
- Ammonium formed (>59 mg L^{-1}) during cultivations did not inhibit biomass production.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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I love you

Tampereen teknillinen yliopisto
PL 527
33101 Tampere

Tampere University of Technology
P.O.B. 527
FI-33101 Tampere, Finland

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