Title: Anaerobic ureolysis of source-separated urine for NH₃ recovery enables direct removal of divalent ions at the toilet

Running title: Urine hydrolysis

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ABSTRACT

Source-separated urine is of interest for nutrient recovery. Most nitrogen recovery technologies rely on ammonia (NH_3) as input, which requires ureolysis. As urease positive bacteria are widespread, source-separated urine is unstable, not only leading to NH_3 release but also loss, odor nuisance, and downstream scaling. Hence, ureolysis ideally occurs in a closed controlled environment close to the toilet. We characterized microbial-induced ureolysis, subsequent divalent cation precipitation, and fermentation in anaerobic sequencing batch reactors (SBRs) at 15°C and 28°C. Temperatures were a proxy for urine hydrolysis in a wet well at street level or in the toilet, respectively. The need for inoculation and the metabolic stability was assessed by inoculation with autofermented urine or a mixture of anaerobic digestion and fermentation sludge. The highest specific ureolysis rates in the SBRs were achieved at 28°C: 2107 ± 395 and 1948 ± 1121 mg N g VSS⁻¹ d⁻¹, for the mixed and autofermented inoculum, respectively. For Ca^{2+} and Mg^{2+} precipitation, and organics fermentation, autofermented urine at 28°C performed best with 47.9 ± 16.4 mg Ca^{2+} g VSS⁻¹ d⁻¹, 8.2 ± 4.6 mg Mg^{2+} g VSS⁻¹ d⁻¹, and 623 ± 129 mg VFA-COD g VSS⁻¹ d⁻¹, respectively. This indicates the hydrolysis reactor should be close to the toilet. The selected inoculum did not impact ureolysis, whereas both Ca^{2+} and Mg^{2+} precipitation and fermentation were better in the SBRs with autofermented urine. Ureolysis was identified as the only process significantly impacting the microbial community, indicating external inoculation would not be required. A urine hydrolysis reactor in the toilet without external inoculation could thus serve as a controlled environment to release NH_3 and remove divalent cations to prevent scaling in downstream transport and processing. For practical implementation in a household toilet, the reactor should

47 be designed for user-friendly precipitate discharge and odor control.

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50 **KEY WORDS**

51 Precipitation; resource recovery; scaling; source-separation; ureolysis; urine

52 hydrolysis

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1. INTRODUCTION

Source-separation of human urine is becoming increasingly important, not only towards resource recovery, but its separation also facilitates downstream wastewater treatment. Urine constitutes only 1% of the volume in domestic wastewater but covers most of the nutrients (Larsen and Gujer 1996). With 4-14 g urea-N L⁻¹ and 0.35-2.5 g P L⁻¹ (Rose et al. 2015), urine is highly suitable for resource recovery. Current resources for nitrogen (N₂ via Haber-Bosch) and phosphorous (mining P rock) for fertilizer production come with high process costs (12.5 kWh kg⁻¹ N and 8.1 kWh kg⁻¹ P as primary energy (Maurer et al. 2003)) and environmental burdens (e.g. exceeding planetary boundaries for N and P (Rockstrom et al. 2009), P scarcity (Cordell et al. 2009), hypoxic zones (Bennett et al. 2001), radioactive waste from phosphate rock mining (Saueia et al. 2005)). Recovery from urine as a readily and worldwide available resource could contribute to solving these issues (Larsen 2011). Aside from resource recovery, source-separation of urine could reduce water use via waterless or vacuum toilets. Consumers will be more open to reuse (grey)water if not contaminated with urine and fecal matter (Larsen et al. 2016). Discharge of untreated nutrient-rich wastewater, e.g. due to leaks in old sewer pipes or combined sewer overflows, could be reduced and subsequently reduce the consequences of excess nutrients in the environment (e.g. hypoxia zones). Many microorganisms present in wastewater can produce urease (Mobley and Hausinger 1989), the enzyme that induces ureolysis (Eq. 1). The subsequent pH rise to the pKa of NH₄⁺/NH₃ (9.25 at 25°C) causes precipitation (scaling), primarily comprised of phosphates (struvite and hydroxyapatite) and carbonates (calcite)(Udert et al. 2003b). The organics present in urine fuel bacterial growth and

thus ureolysis.

This addresses the main issue in source-separation: urine instability due to ureolysis. As urine may not be sterile in the urinary tract and is further contaminated when leaving the human body (Hoglund et al. 1998, Wolfe and Brubaker 2015), uncontrolled ureolysis causes nitrogen loss (NH_3 volatilization), malodors, and undesired scaling. Stabilization of urine at the toilet for transport and centralized resource recovery can be considered a necessary step. Although extensively studied (e.g. sterilization by addition of acid (Hellstrom et al. 1999) or base (Randall et al. 2016), nitrification (Bischel et al. 2015), bio-electro-Fenton system (Zhou et al. 2018)), stabilization always comes with an energy or chemical cost. A sustainable and manageable method for stabilization at the toilet is therefore still not established. As different technologies currently in development for resource recovery from urine consider ureolysed urine ($\text{NH}_4^+/\text{NH}_3$) as the input (Antonini et al. 2011, Arredondo et al. 2017, Christiaens et al. 2017, Fumasoli et al. 2016, Kuntke et al. 2014, Ledezma et al. 2017, Luther et al. 2015, Pronk et al. 2006, Tarpeh et al. 2018, Zhao et al. 2013), alternative would be to establish controlled ureolysis as close as possible to the source, the toilet. This would create a stream low in Ca^{2+} and Mg^{2+} , amenable for recovery options. Nutrient recovery close to the toilet could avoid the implementation of a second expensive sewer network (Randall and Naidoo 2018). Technologies should therefore be robust, easy to operate by consumers, have low maintenance requirements, and deliver products that can be readily used by the consumer (e.g. water, fertilizer, or food additives).

In this study, urine hydrolysis is evaluated under different anaerobic conditions in SBRs to engineer it into a controlled process. Ureolysis, divalent cation removal, and organics fermentation are quantified at 15°C and 28°C as a proxy for urine hydrolysis in a wet well at street level in a temperate climate or in the toilet, respectively. The temperature in the toilet was set at 28°C based on a body temperature of around 36-37°C while taking into account temperature loss during urinating and during the residence time in the toilet with an ambient room temperature of 21°C. The need for inoculation and the metabolic stability of the hydrolysis tank is studied by inoculation with autofermented urine (AUTO) or mixed anaerobic ureolytic sludge (MIX), at the different temperatures. The main objective was to identify the best position and the potential need for inoculation of the urine hydrolysis reactor to obtain complete ureolysis, maximum Ca^{2+} and Mg^{2+} precipitation without addition of caustic, and potential recovery of fermentation products.

2. MATERIALS AND METHODS

2.1 Urine and inoculum

Urine from male donors was collected throughout the day via a waterless urinal at Ghent University with permission of the university's ethical committee. The fresh urine was stored for maximum 2 days at 4°C until use (Table 1). As a precaution for nitrogen and carbon losses due to aerobic microbial processes, residual oxygen was removed from the 20L feed vessel before connection to the reactors, by sparging with N_2 for 20 minutes. A N_2 filled gas bag was connected to maintain atmospheric pressure in the vessel.

One inoculum, hereafter called MIX, was a mixture of one volume unit alkaline

fermentation sludge and three units of anaerobic digestion sludge (Table 1). The inoculum was preconditioned at 15°C and 28°C during 12 days before reactor inoculation. The other inoculum, hereafter called AUTO, was autofermented urine at 15°C and 28°C for 12 days.

Table 1 Average and standard deviation (SD) characteristics of men's, fresh urine (n = 4), alkaline fermentation sludge, and anaerobic digestion sludge (n = 3)

Parameter	Unit	Fresh urine ^a	Anaerobic digestion sludge	Fermentation sludge
TS	g kg ⁻¹	n.d.	49.37 ± 0.52	47.36 ± 0.62
VS	g kg ⁻¹	n.d.	32.18 ± 0.53	22.78 ± 0.42
pH	-	7.35 ± 0.12	8.87 ± 0.02	10.23 ± 0.01
EC	mS cm ⁻¹	20.64 ± 1.28	25.83 ± 0.81	22.27 ± 0.12
TKN	mg N L ⁻¹	5268 ± 304	4488 ± 209	n.d.
TAN	mg N L ⁻¹	1250 ± 216	2241 ± 102	737 ± 144
Total COD	mg O ₂ L ⁻¹	5600 ± 1237	1163 ± 78	1126 ± 85
Soluble COD	mg O ₂ L ⁻¹	n.d.	1039 ± 106	852 ± 124
VFA	mg O ₂ L ⁻¹	248 ± 62	331 ± 24	1742 ± 89
Na ⁺	mg L ⁻¹	2018 ± 195	1269 ± 58	6310 ± 517
K ⁺	mg L ⁻¹	1597 ± 126	1255 ± 71	263
Ca ²⁺	mg L ⁻¹	58 ± 10	n.d.	n.d.
Mg ²⁺	mg L ⁻¹	11 ± 4	n.d.	n.d.
Cl ⁻	mg L ⁻¹	3650 ± 139	2586 ± 71	121 ± 8
NO ₂ ⁻	mg N L ⁻¹	<LOQ ^b	5.64 ± 8.07	<LOQ ^b
NO ₃ ⁻	mg N L ⁻¹	8.65 ± 5.15	6.88 ± 6.79	<LOQ ^c
PO ₄ ³⁻	mg L ⁻¹	676 ± 102	314 ± 19	957 ± 37
SO ₄ ²⁻	mg L ⁻¹	810 ± 62	30 ± 12	165 ± 3

n.d. = not determined

^aLimited precipitation initiated already in the collection phase and could explain the slightly higher pH, lower magnesium concentrations, and slightly variable phosphate concentrations. However, the low magnesium concentrations were additionally

verified by separately collecting fresh urine in small recipients and analyse them directly with an AAS device. Equally low values were measured.

^bBelow quantification limit (LOQ) of 3 mg NO₂⁻ -N L⁻¹

^cBelow quantification limit (LOQ) of 2.3 mg NO₃⁻ -N L⁻¹

2.2 Experimental set-up

Urine fermentation as a pretreatment for nitrogen recovery technologies was studied in sequencing batch reactors (SBR). Each reactor consisted of a 1 L glass bottle with a working volume of 0.5 L, closed with a butyl rubber stopper. Three tubes went through the stopper, one for feed, one for sludge sampling at the bottom, and one for effluent at the level of 300 mL (from day 54: 400 mL). A needle was pierced through the stopper to connect a 0.5 L N₂ filled gas bag to maintain atmospheric pressure. Peristaltic feed and effluent pumps (Watson Marlow, Ghent, Belgium) controlled by timers connected the reactors to one feed (stored at 4°C) and effluent vessels by means of gastight tubing (Tygon food, Saint-Gobain, USA). Reactors were operated at 15°C in a temperature controlled room and at 28°C in a water bath in the same laboratory. All reactors, including the water bath, were placed on a shaker operated at 80 rpm.

2.3 Reactor operation

The combined effect of temperature (15 and 28°C) and inoculum (AUTO and MIX) on ureolysis, Ca²⁺ and Mg²⁺ precipitation, and fermentation was studied in SBRs to mimic discontinuous urination in a toilet. Four treatments were run in duplicate SBRs: 15AUTO, 15MIX, 28AUTO, and 28MIX. To achieve small and high-rate systems, a volumetric loading rate of 10.7 ± 1.1 g N L⁻¹ d⁻¹ was applied at a hydraulic retention

time (HRT) of 0.41 ± 0.07 days. Bacterial sludge and precipitates were allowed to build up. One SBR cycle consisted of a 10' fill phase, 2 h reaction time, 1' settling, 4' draw phase, and a 9' idle phase to have 5 cycles per HRT (from day 54: 5' draw and 8' idle). At start-up, reactors were filled with 10% inoculum and 90% tap water. Reactors were continuously operated, except over the weekend for the first 30 days due to limited urine availability. On day 54, pump issues were solved and a constant reactor volume could be maintained. Steady state (i.e., 3 HRTs) was achieved at day 56 and lasted till the end of the experiment (day 145). Sampling of the influent tank occurred upon refilling every 2-3 days. Reactor effluent and headspace composition were sampled 3 times a week while reactor solids were sampled once a week after rigorous mixing. The activity occurring in the influent tubes of the SBRs was quantified in a tubing test. Liquid was sampled from the influent tubing for all SBRs during two fill phases and after the reaction time period to determine ureolysis, precipitation, and fermentation percentages.

2.4 Batch activity tests

To evaluate reactor performance, on day 145, reactor sludge was taken after thorough mixing to quantify ureolysis, precipitation, and fermentation rates in an anaerobic batch test. From each reactor, duplicate 1L glass bottles were set up with 70 mL reactor mixed liquor and 630 mL fresh urine. One biomass control flask per reactor was set up with 70 mL reactor mixed liquor and 630 mL centrifuged reactor effluent. One substrate control per temperature consisted of 700 mL fresh urine. Bottles were incubated in the dark at 15°C or 28°C and shaken at 80 rpm. Headspace pressure was measured before gas and subsequent liquid sampling. The total sampling volume over the 320 h test was around 50 mL. At the end of the test,

precipitate was sampled, dried, and stored at room temperature for characterization by x-ray diffraction.

Time series of concentrations and activity percentages were obtained, to which models were fitted with the packages `grofit` (version 1.1.1.1) for logistic models, `FME` (version 1.3.5) for Monod models, and `minpack.lm` (version 1.2.1) for exponential models in R (version 3.3.1). Models gave insight in maximum activity rates and efficiencies, i.e., the maximum slope of a concentration and activity percentages series, respectively, and times at which plateau efficiencies were reached. More information and a visual representation can be found in SI (section E + Fig. E.1).

2.5 Chemical analyses

Liquid samples were centrifuged for 5' at 10 000 rpm and 0.2 μm filtered prior to analysis, except for total chemical oxygen demand (tCOD). Samples were analysed for pH, electrical conductivity (EC), Ca^{2+} and Mg^{2+} via atomic absorption spectrometry, and monovalent cations (Na^+ , total ammonium nitrogen (TAN), K^+), anions (Cl^- , NO_2^- , NO_3^- , PO_4^{3-} , SO_4^{2-}), and volatile fatty acids (VFA)(lactate, formate, acetate, propionate, and butyrate) via ion chromatography. Inoculum VFA were measured with a gas chromatograph. Kjeldahl nitrogen (TKN), total (suspended) solids (T(S)S), and volatile (suspended) solids (V(S)S) were quantified according to standard methods (Greenberg 1992) while nanocolor kits (Macherey-Nagel, Germany) were used to quantify total and soluble chemical oxygen demand (tCOD and sCOD). Headspace pressure was quantified with a tensiometer and gas sample composition with compact gas chromatography. Precipitate samples were characterized by powder X-ray diffractometry (XRD). Detailed information about chemical analyses can be found in SI (section A). Calculations are provided in detail

in SI (sections D and E).

2.6 Molecular analyses and data processing

Liquid samples were centrifuged for 10' at 12 000 rpm and the pellet was stored at -20°C. The DNA was extracted following the method (Vilchez-Vargas et al. 2013). The quality of the DNA extracts was validated through agarose gel electrophoresis. A control PCR was carried out using the primers P338 and P518 (Muyzer et al. 1993), following the protocol of Boon et al. (2002). The PCR product quality was verified *via* agarose gel electrophoresis to confirm that no PCR inhibition took place. The DNA extracts were sent to LGC Genomics GmbH (Berlin, Germany), where Illumina sequencing was carried out on the MiSeq platform, as described in SI (section B). The raw fastq files that were used to create the OTU table, which served as a basis for the microbial community analysis, have been deposited in the National Center for Biotechnology Information (NCBI) database (Accession number SRP131381). Further data processing is described in SI (section B).

3. RESULTS

3.1 The highest ureolysis efficiency was achieved at 28°C in SBRs

Ureolysis and, to a limited extent, organics conversion to easily degradable VFA, are the main pretreatment processes making resources available for downstream upgrading and product recovery. Calcium and magnesium precipitation avoid scaling in downstream transport and processes. The influence of temperature and inoculum on these processes was studied in SBRs. Prior to the experiments described here, a preliminary test was performed showing a faster ureolysis for aerobic than for anaerobic urine pretreatment (SI section C). However, the closed reactor set-up used for anaerobic conditions creates the considerable advantage to prevent nitrogen losses, making it preferable in this context.

Ureolysis can be quantified by the evolution of TAN relative to TKN (i.e., the sum of TAN and organic nitrogen) as the maximal urine nitrogen concentration (Figure 1). It is important to note that urine contains other nitrogen compounds such as protein, although urea accounts for about 90% of TKN in urine (Rose et al. 2015). Mixed inoculation improved ureolysis to $63 \pm 8\%$ and $54 \pm 7\%$, at 28 and 15°C, respectively, on top of the $24 \pm 5\%$ that occurred in the cooled influent tank (Table 2). The influent tubing test indicated that ureolysis in the influent tubing was limited (max. $2.3 \pm 0.6\%$). Correcting for biomass concentrations shows that treatments at 28°C achieved the highest specific activity rates, irrespective of the inoculum (Table 2). Ureolysis introduced a rapid pH increase which is, already at low TAN levels, strongly buffered around the pKa of ammonium (9.25 at 25°C). Both FA and EC have a positive correlation with ureolysis efficiency (SI Fig. D.1). A Spearman correlation test yielded 0.69 and 0.78, respectively, with $P < 0.05$.

Figure 1 Stacked average ($\pm$ standard deviation, SD) steady state organic N and TAN (i.e., NH_4^+ and NH_3) concentrations (mg N L^{-1}) indicate highest ureolysis levels for the SBRs operated at 28°C with mixed inoculum (28MIX), compared to the other treatments at 15°C and/or with autofermented urine as inoculum (AUTO), irrespective of a 24% influent ureolysis level. The reactor pH and reactor temperature determine free ammonia (FA) concentrations. Limited TKN losses occurred. ($n = 12$, i.e., 6 steady state datapoints per duplicate treatment)

3.2 The highest Ca^{2+} and Mg^{2+} removal efficiency was achieved in SBRs inoculated with autofermented urine

The pH increase also caused precipitation of Ca^{2+} and Mg^{2+} salts with phosphate, present in urine, and bicarbonate, released during ureolysis (Figure 2). Temperature had the strongest impact on Ca^{2+} removal as treatment 28AUTO achieved the highest efficiency of $78 \pm 6\%$, followed by 28MIX with $58 \pm 0\%$ (Table 2). The same trend is observed after correcting for biomass concentrations (Table 2). Magnesium removal efficiencies were all similar (90 ± 8 to $94 \pm 4\%$), whereas the specific activity rates improved for AUTO treatments, irrespective of temperature (Table 2). The influent tubing test indicated a maximum of $4.3 \pm 3.3\%$ Ca^{2+} removal in the influent tube and no detectable Mg^{2+} removal, except for 28AUTO with $36.6 \pm 15.4\%$.

Figure 2 Average ($\pm$ SD) steady state Ca^{2+} and Mg^{2+} concentrations (mg L^{-1}) indicate highest precipitation, especially for Ca^{2+} , for the SBRs operated at 28°C with autofermented inoculum (28AUTO) compared to the other treatments at 15°C and/or with mixed inoculum (MIX). ($n = 12$, i.e., 6 steady state datapoints per duplicate treatment)

3.3 Fermentation in SBRs was favoured at 28°C

The anaerobic conditions in the closed reactor set-up allowed fermentation of urine organics (Figure 3). Fermentation was favoured by higher temperatures, as well as by the mixed inoculum (Table 2). Treatment 28MIX achieved the highest VFA production efficiency, converting $17 \pm 9\%$ of the initial COD to VFA, mostly acetate ($87 \pm 3\%$). Specific activity rates confirmed the importance of temperature, but indicated AUTO as the inoculum with the highest fermentation activity (Table 2). Fermentation in the influent tank or natural presence of measured VFA in fresh urine contributed an additional $5 \pm 1\%$ to all SBRs while in the influent tubing test no fermentation could be observed. Losses of tCOD to CH_4 (maximum $17 \text{ mg CH}_4 \text{ L}^{-1}$ at STP) and H_2 (maximum $0.22 \text{ mg H}_2 \text{ L}^{-1}$ at STP) were limited. No biomass wash-out could be observed since total and soluble COD of the effluent were similar. A maximum of 1 g VSS L^{-1} of biomass accumulation was observed, for treatment 28MIX, which theoretically resembles to $1.33 \text{ g COD L}^{-1}$ loss to biomass.

Figure 3 Average ($\pm$ SD) steady state tCOD, VFA, and acetate (Ac^-) concentrations (mg COD L^{-1}) indicate highest fermentation for the SBRs operated at 28°C with mixed inoculum (28MIX) compared to the other treatments at 15°C and/or with autofermented inoculum (AUTO). Limited COD losses occurred. (VFA: $n = 12$, i.e., 6 steady state datapoints per duplicate treatment; tCOD: $n = 8$, i.e., 4 steady state datapoints per duplicate treatment)

Table 2 Average ($\pm$ SD) comparison between steady state SBR performance and the batch activity test

Parameter	Treatment	Specific activity rate ($\text{mg g VSS}^{-1} \text{d}^{-1}$)		Volumetric activity rate ($\text{mg L}^{-1} \text{d}^{-1}$)		Efficiency (%)		HRT (d)	
		SBR ¹	Batch ^{2,4}	SBR ¹	Batch ^{2,4}	SBR	Batch ^{2,4}	SBR	Batch ^{3,4}
Ureolysis (TAN)	15AUTO	1379 $\pm$ 660	3203 $\pm$ 507	3504 $\pm$ 2156	2046 $\pm$ 323	29 $\pm$ 25	76 $\pm$ 4	0.36 $\pm$ 0.05	3.17 $\pm$ 0.73
	15MIX	932 $\pm$ 200	3941 $\pm$ 1278	5288 $\pm$ 904	4227 $\pm$ 1370	54 $\pm$ 7	80 $\pm$ 7	0.40 $\pm$ 0.07	1.60 $\pm$ 0.34
	28AUTO	1948 $\pm$ 1121	7139 $\pm$ 1437	4624 $\pm$ 2081	4614 $\pm$ 929	40 $\pm$ 17	91 $\pm$ 5	0.44 $\pm$ 0.08	1.54 $\pm$ 0.53
	28MIX	2107 $\pm$ 395	7593 $\pm$ 2609	7294 $\pm$ 1156	5562 $\pm$ 1911	63 $\pm$ 8	82 $\pm$ 9	0.45 $\pm$ 0.07	1.34 $\pm$ 0.34
Ca^{2+}	15AUTO	25.0 $\pm$ 13.3	15 $\pm$ 6	62 $\pm$ 29	10 $\pm$ 4	49 $\pm$ 10	74 $\pm$ 3	0.36 $\pm$ 0.05	2.72 $\pm$ 0.11
	15MIX	9.1 $\pm$ 3.4	3 $\pm$ 0	54 $\pm$ 18	3 $\pm$ 0	50 $\pm$ 9	67 $\pm$ 3	0.40 $\pm$ 0.07	3.17 $\pm$ 0.87
	28AUTO	47.9 $\pm$ 16.4	55 $\pm$ 13	97 $\pm$ 21	36 $\pm$ 8	78 $\pm$ 6	82 $\pm$ 1	0.44 $\pm$ 0.08	1.54 $\pm$ 0.15
	28MIX	23.2 $\pm$ 8.2	27 $\pm$ 2	74 $\pm$ 21	20 $\pm$ 1	57 $\pm$ 10	74 $\pm$ 2	0.45 $\pm$ 0.07	1.39 $\pm$ 0.09
Mg^{2+}	15AUTO	6.0 $\pm$ 2.6	5 $\pm$ 4	20 $\pm$ 8	3 $\pm$ 2	92 $\pm$ 7	86 $\pm$ 5	0.36 $\pm$ 0.05	1.68 $\pm$ 0.33
	15MIX	2.8 $\pm$ 1.2	1 $\pm$ 1	19 $\pm$ 7	1 $\pm$ 1	94 $\pm$ 4	60 $\pm$ 7	0.40 $\pm$ 0.07	0.15
	28AUTO	8.2 $\pm$ 4.6	3 $\pm$ 1	21 $\pm$ 8	2 $\pm$ 1	90 $\pm$ 8	82 $\pm$ 7	0.44 $\pm$ 0.08	0.66 $\pm$ 0.42
	28MIX	5.5 $\pm$ 2.6	0 $\pm$ 0	22 $\pm$ 8	0 $\pm$ 0	93 $\pm$ 5	61 $\pm$ 10	0.45 $\pm$ 0.07	0.20
Fermentation (VFA-COD)	15AUTO	172 $\pm$ 63.9	734 $\pm$ 274	355 $\pm$ 266	469 $\pm$ 175	4 $\pm$ 2	20 $\pm$ 2	0.36 $\pm$ 0.05	7.30 $\pm$ 5.33
	15MIX	177 $\pm$ 59.6	1333 $\pm$ 1630	1148 $\pm$ 365	1429 $\pm$ 1748	11 $\pm$ 4	34 $\pm$ 2	0.40 $\pm$ 0.07	5.19 $\pm$ 4.26
	28AUTO	623 $\pm$ 129	1979 $\pm$ 866	1360 $\pm$ 251	1279 $\pm$ 560	12 $\pm$ 3	27 $\pm$ 1	0.44 $\pm$ 0.08	3.44 $\pm$ 1.03
	28MIX	519 $\pm$ 168	4277 $\pm$ 2094	1995 $\pm$ 776	3133 $\pm$ 1534	17 $\pm$ 9	32 $\pm$ 2	0.45 $\pm$ 0.07	3.67 $\pm$ 2.88

¹ Steady state values ($n = 12$, i.e., 6 steady state datapoints per duplicate treatment)

² Values obtained by fitting models (SI section E) to measured time series of concentration to obtain activity rates, i.e., the maximum slope, and time series of efficiency to obtain the maximum efficiency, i.e., the plateau reached by the fitted curve

³ HRT in the batch test was defined as the minimum time to reach 95% of the efficiency plateau.

⁴ Standard deviations considered both model and replicate standard deviations (efficiency, ureolysis and fermentation rates) or only replicates (HRT, Ca^{2+} and Mg^{2+} removal rates)

3.4 Batch activity test showed similar trends as SBRs

To characterize the maximum ureolysis, precipitation, and fermentation rates, as well as efficiencies and the times at which these would be reached, a batch test was conducted and models were fitted to the obtained time series of concentrations and activity percentages (SI Fig. E.1).

Overall, similar trends between the batch test and SBRs were observed, irrespective of volumetric or specific activity rates. The equal or lower volumetric activity rates in the batch test compared to the SBRs were due to lower biomass concentrations (SI Fig. D.2 and Fig. E.2) and higher substrate to biomass ratio's (i.e., 20/80 in SBRs and 90/10 in batch). When comparing the different batch tests, 15MIX contained more biomass ($1072 \pm 269 \text{ mg VSS L}^{-1}$) than the other treatments (639 ± 233 to $732 \pm 185 \text{ mg VSS L}^{-1}$).

For ureolysis in batch, temperature increased activity rates and efficiencies more than the type of inoculum (Table 2, SI Fig. E.1). Interestingly, specific activity rates such as for 28MIX, were considerably higher in batch ($7593 \pm 2609 \text{ mg N g VSS}^{-1} \text{ d}^{-1}$) than in the SBRs ($2107 \pm 395 \text{ mg N g VSS}^{-1} \text{ d}^{-1}$). The maximum activity rate measured in the batch tests always occurred within 50 hours where parameters like pH, EC, and FA did not yet reach values similar as in the SBRs in steady state operation. The time to reach 95% of the maximum efficiency in batch (SI Fig. E.1), which was similar to the efficiency in the SBRs (including 24% ureolysis in the influent tank), i.e., the HRT in Table 2, lasted longer in batch (1.34 ± 0.34 days for 28MIX) compared to the SBRs (0.45 ± 0.07 days) and can be attributed to the higher substrate to biomass ratio in batch.

For Ca^{2+} precipitation, temperature improved removal rates and efficiencies, and was

348 additionally enhanced by autofermented urine (Table 2). Mg^{2+} precipitation was
349 improved by autofermented urine and, in contrast to the SBRs, preferable at lower
350 temperatures. Specific Ca^{2+} removal rates were similar in batch as in the SBRs while
351 for Mg^{2+} they were higher in the SBRs. As for ureolysis, the time to reach 95% of the
352 maximum Ca^{2+} removal efficiency was longer, 1.54 ± 0.15 days for 28AUTO,
353 compared to 0.45 ± 0.07 days in the SBR. However, final efficiencies were similar,
354 likely due to the high substrate to biomass ratio in batch and thus slower ureolysis
355 and carbonate release. For Mg^{2+} , despite a similar time in batch as in the SBRs,
356 removal efficiencies of the SBRs could not be achieved. The main crystal precipitate
357 (>77%) in all batch test bottles was identified as struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$)
358 according to the XRD results. For 15MIX, the remaining precipitate was identified as
359 calcite (CaCO_3) (10%).

360 Fermentation activity rates and efficiencies strongly increased with temperature, and
361 were higher for a mixed inoculum at 28°C (Table 2). As for ureolysis, specific activity
362 rates in batch such as for 28MIX, were considerably improved compared to the
363 SBRs, probably due to more favorable initial physicochemical conditions. Despite the
364 longer time to reach 95% of the maximum fermentation efficiency, 3.67 ± 2.88 days
365 for 28MIX compared to the HRT of 0.45 ± 0.07 days in the SBR, the fermentation
366 efficiency almost doubled compared to the SBRs, although the 28MIX SBRs
367 contained 3.12 ± 0.13 to 4.19 ± 0.13 g VSS L^{-1} at steady state compared to $0.73 \pm$
368 0.19 g VSS L^{-1} in batch.

370 **3.5 Microbial community composition significantly influenced by ureolysis, and** 371 **clustered together according to temperature and inoculum**

372 The illumina 16S rRNA gene amplicon sequencing data were analysed for

373 differences between the three independent variables applied in the SBRs: type of
374 inoculum (AUTO or MIX), temperature (15°C or 28°C), and time (T1 and T2, i.e., at
375 the start and end of steady state, respectively, with 82 days in between). Canonical
376 correspondence analysis identified conductivity, pH, and free ammonia (FA) - all
377 related to the ureolysis process - as the key parameters that affect the microbial
378 community (Figure 4). As the direction of the arrows is similar, one parameter will
379 increase with the other. Indeed, ureolysis comes with elevated and interlinked pH,
380 conductivity, and FA levels. The arrow length is also similar, indicating that the
381 microbial community seems to be equally impacted by the three parameters, and
382 thus would only significantly shift due to ureolysis. Data points of different
383 temperature and to a lesser extent inoculum treatments cluster together, while time is
384 more scattered. This was statistically confirmed in the NMDS plots ($P < 0.05$)(SI Fig.
385 D.3), but could not be linked to individual significantly different OTUs by a DESeq2
386 analysis. The community would thus change as a whole related to ureolysis.

387 For all independent variables inoculum, temperature, and time, most of the OTUs
388 with highest relative abundance were all classified under the families
389 *Carnobacteriaceae*, *Alcaligenaceae*, an unknown family XI classified under the order
390 of *Clostridiales*, and *Enterobacteriaceae*. Different temperature treatments had a
391 more pronounced effect on the microbial community composition than the inocula
392 (Figure 5, SI Fig. D.4). About 50% of total relative abundance could be explained by
393 the OTUs belonging to the aforementioned families for AUTO, MIX, and 15°C, while
394 for 28°C this was only 30%. A significantly ($P < 0.05$) higher relative abundance for
395 several OTUs between the different clusters. In autofermented urine, OTUs classified
396 within the genera *Providencia*, *Alcaligenes*, *Aerococcus*, and *Vagococcus* were
397 significantly higher abundant than for the mixed inoculum, while for the mixed

inoculum compared to autofermented urine, this was *Tissierella*, *Thiopseudomonas*, *Proteiniphilum*, *Providencia*, and three OTUs belonging to the order of the *Clostridiales*. For both temperature clusters, genus *Providencia* and family *Carnobacteriaceae* had a significantly different relative abundance. At 15°C, genus *Oblitimonas* was significantly more abundant while at 28°C this was *Oligella* and *Aerococcus*. Genus *Thiopseudomonas* was significantly more abundant at T1 compared to genus *Paenalcaligenes* at T2.

Figure 4 Canonical Correspondence Analysis plots of all SBRs with two steady state data points (T1 and T2) show clustering for inoculum and temperature, but not for time. Mainly ureolysis impacted the microbial community (Conductivity ($P < 0.001$), pH ($P < 0.001$) and Free ammonia ($P < 0.003$)).

Figure 5 Relative abundance of OTUs per cluster (i.e., AUTO/MIX, 15/28°C, T0/1/2) with cut-off at 1% average relative abundance. An asterisk (*) indicates the significantly different OTUs between the clusters ($P < 0.05$) as determined by the DESeq2 analysis.

3.6 Reactor microbial diversity impacted by the inoculum

Microbial community diversity differences between the clusters AUTO/MIX, 15/28°C, and timepoints T0, T1 and T2, was calculated by means of the Hill numbers. For both temperature and time, there was no significant difference ($P < 0.05$) between 15 or 28°C, and times 0, 1, and 2, respectively, for three Hill numbers (SI Fig. D.5, D.7). This means that the average richness and evenness of the communities was not significantly influenced by temperature nor time. However, the initial inocula (T0)

showed higher variability whereas this converges to the average values in later time points (SI Fig. D.7).

Inoculum, on the other hand, has a significant and not unexpected impact on community richness, based on Hill number 0 (SI Fig. D.6). The MIX reactors had a higher richness (158 ± 17) than the AUTO SBRs (115 ± 28). In addition, Hill numbers 1 were also significantly different (27 ± 10 versus 19 ± 5 , for MIX and AUTO inoculum, respectively, $P < 0.05$), indicating that the microbial community in the MIX inoculum in the SBRs had a higher diversity. No significant difference could be observed in Hill number 2 indicating that the evenness was not different between MIX (14 ± 7) and AUTO (11 ± 2). If diversity and more specifically different phylogenetic species indicate a more diverse metabolic potential, the mixed reactors could be more functionally redundant to negative impacts, e.g. antibiotics in the influent.

4. DISCUSSION

4.1 Urine hydrolysis likes it warm

Source-separation must deal with the instability of urine and needs to foresee a pretreatment step before resource recovery of the nutrients is possible. The most straight forward strategy combining both, is the decentralized hydrolysis of urine in a gravity-fed system without pumps. Another option would be urine stabilisation by the addition of $\text{Ca}(\text{OH})_2$ (Randall et al. 2016) or ash (Senecal and Vinneras 2017). However, both hydrolysed and stabilised urine require a decentral storage tank until the urine is collected. For hydrolysed urine, gastight systems are required but these are widely used in industry for the transport and storage of corrosive chemicals, e.g. HCl. For downstream and more central nutrient recovery, urine hydrolysis is required to, amongst others, release ammonia. In this respect, the stabilised urine will need additional acid chemicals to decrease the pH and re-activate the bacteria for hydrolysis. On the other hand, the alkalified urine could be concentrated without hydrolysis, for use as fertilizer (Xu et al. 2017).

For decentralized hydrolysis, two temperatures, 15 and 28°C, were studied as representatives for a hydrolysis tank on street level (so called 'sewer wet wells') or in the toilet, respectively. Ureolysis, Ca^{2+} precipitation, and fermentation rates and efficiencies were positively influenced by the higher temperature.

For ureolysis, this was in line with previous studies (Liu et al. 2008, Zhang et al. 2013). However, volumetric activity rates were 1.5 and 3 times lower in batch experiments resembling our AUTO experiments at 28°C and 15°C, respectively. The lack of mixing in these reports might explain this discrepancy as the test set-up was similar, with 10% stale urine as inoculum (Liu et al. 2008). The time to complete

470 ureolysis ranged from 2 (Liu et al. 2008) to 3 (Zhang et al. 2013) days at 25°C and
471 28°C, respectively, whereas we observed 1.54 days for 28AUTO. Even at 0°C
472 urease can be active (Mobley and Hausinger 1989), indicating that urease present in
473 urine from bacteria living in the urinary tract could already induce ureolysis and
474 scaling in the cooled (4°C) influent tank. If ureases are present in urine, immediate
475 pretreatment is required favoring a hydrolysis tank in the toilet, instead of at street
476 level.

477 Precipitation of Ca^{2+} and Mg^{2+} in the batch tests followed an exponential 1st order
478 kinetic without lag phase, indicating immediate precipitation. Additional tests with
479 higher time and sampling resolution might be needed to confirm this because these
480 results contrast with (Udert et al. 2003a), who reported 45 minutes before
481 magnesium struvite as the first crystal started precipitating in pipes with an active
482 ureolytic biofilm at 25°C. Regardless of the exact time of precipitation onset, the
483 shorter the pipes to the hydrolysis tank, the lower the chance for scaling due to Ca^{2+}
484 and Mg^{2+} precipitation, and the lower the maintenance costs. The HRT of wastewater
485 in the sewer pipes to a street wet well is in the order of minutes and might be shorter
486 compared to the precipitation time. However, without extensive flushing, always
487 some urine will remain in the pipes, allowing for retention times long enough to
488 induce precipitation.

489 The main precipitation products found in the batch tests were struvite and calcite, in
490 contrast to Udert et al. (2003b), who reported struvite and calcium phosphate.
491 Calcium precipitation as calcite is an endothermic process requiring high
492 temperatures (Garrels and Christ 1965). This explains the overall higher precipitation
493 activity rates and efficiencies at 28°C. Additionally, precipitation was enhanced at
494 alkaline pH, as observed in our experiments (Garrels and Christ 1965).

495 Fermentation processes are normally conducted at 35°C (mesophilic) or 55°C
496 (thermophilic) because hydrolysis and fermentation rates are enhanced at higher
497 temperatures (Yu and Fang 2003). The goal was to explore to which extent VFA
498 were produced in an ureolysis reactor for potential recovery. Specific production
499 rates in our 28MIX SBRs were similar to the scarce literature on liquid waste
500 fermentation achieving 723 and 552 mg COD g VSS⁻¹ d⁻¹ at HRTs of 0.5 days,
501 neutral pH, low salinity, and FA concentrations not exceeding 369 mg N L⁻¹ (Demirel
502 and Yenigun 2004, Yu and Fang 2003). This is surprising given the harsh
503 environment for fermenters, in the ureolytic reactors, with alkaline and salty
504 conditions and FA concentrations of 2523 ± 193 mg N L⁻¹ potentially causing toxicity
505 on fermenting micro-organisms (Niu et al. 2013). Despite our robust ureolytic
506 fermenting community, organics fermentation was incomplete and the VFA
507 production efficiency could be partially increased by prolonging the HRT (Zhang et al.
508 2013), as shown in our batch test. This would however lower the production rates
509 even further. A trade-off between the importance of fermentation, ureolysis, and
510 precipitation processes needs to be made.

511 Temperature caused a microbial community shift, although there was no influence on
512 richness or evenness. On OTU level, both temperatures 15 and 28°C had only
513 limited significantly different OTUs, but at 28°C, the relative abundance of these was
514 higher. This indicated some species were influenced by the higher temperatures.

515 Functionalities for urine hydrolysis could potentially be attributed to *Aerococcus*
516 which was found in urine (Dong et al. 2011), *Oligella* and *Providencia* which were
517 related to urinary tract infections and ureolysis (Baqi and Mazzulli 1996, dos Santos
518 et al. 2015).

Overall, 28°C gave the better result for ureolysis, Ca^{2+} precipitation, and fermentation rates and efficiencies, indicating that the hydrolysis reactor should preferably be placed in or close to each individual toilet.

4.2 Autofermented urine as inoculum improves ureolysis and precipitation

A different initial microbial community might have an impact on urine hydrolysis. To characterize the need for inoculation and metabolic stability, two types of inocula were studied: autofermented urine (AUTO) and a mixture of anaerobic digestion with fermentation sludge (MIX). Although temperature dominated activity rates in urine hydrolysis, the inoculum type additionally influenced fermentation (MIX), Ca^{2+} precipitation (AUTO), and was even the main influencing factor for Mg^{2+} removal (AUTO). As the main focus of urine pretreatment is on TAN release and scaling prevention, autofermented urine is preferred, thus, no inoculation would be required. However, to avoid a lag-phase to grow bacteria contained within the fresh urine, one might inoculate with urine, stored for one to two weeks at 28°C. If inoculation at start-up of the hydrolysis reactor is considered, the amount of biomass and the ratio substrate to biomass increases the volumetric activity rates for ureolysis and divalent cation removal if more biomass is present. Udert et al. (2003a) confirmed this with increased ureolytic volumetric activity rates in the pipes where more solids were present than in the collection tank. The higher specific activity rates achieved in our batch test could only be reached at start-up in the hydrolysis tank but not during steady state operation, because of deteriorating physicochemical conditions, e.g. alkaline pH, high salinity, free NH_3 , impacting mainly biological processes such as ureolysis and fermentation. Indeed, ureolysis was the only process identified as having a significant impact on the community. Initially, the inocula AUTO and MIX

were different, but partially converged over time with 50% of their community explained by the same OTUs. Nevertheless, the inocula formed separate clusters indicating a different composition. The more dominant OTUs with significantly different relative abundance were amongst others *Tissierella* which is known to degrade creatinine with acetate production (Harms et al. 1998) and was found by Barbosa et al. (2017) in an urine MFC, *Vagococcus* which was found in a swine manure pit (Lawson et al. 2007) and *Proteiniphilum* which ferments proteins (Chen and Dong 2005). Since the mixed inoculum showed significantly higher richness than the autofermented urine, a higher metabolic potential could be present which could result in higher functional redundancy in case of adverse conditions for the microbiome, e.g. antibiotics release in the hydrolysis tank (Allison and Martiny 2008).

4.3 System engineering and practical considerations

A urine hydrolysis tank in a source-separation toilet (and initially inoculated with autofermented urine if a rapid start-up is required), could fulfill the main function of TAN release for downstream recovery and precipitation of Ca^{2+} and Mg^{2+} to prevent scaling. A household with 4 inhabitant equivalents (IE) produces $1 \text{ L IE}^{-1} \text{ d}^{-1}$ at 5 g N L^{-1} (Larsen and Gujer 1996). At an HRT of 12h, the volumetric loading rate would be $10 \text{ g N L}^{-1} \text{ d}^{-1}$ in a reactor of 4 L, including a design safety factor of 2. Given the specific ureolysis rate achieved at 28AUTO, $1.9 \pm 1.1 \text{ g N g VSS}^{-1} \text{ d}^{-1}$, this would imply a biomass concentration of 5.3 g VSS L^{-1} is needed. This concentration also allows Ca^{2+} removal. If organics fermentation is to be pursued for VFA recovery, 20 g VSS L^{-1} would be needed.

To prevent NH_3 loss and accompanying malodor, a vacuum toilet would be preferred.

A design parameter that should be considered for the hydrolysis tank is the need for

569 mixing to maximize contact between urine and the urease-producing bacteria. In our
570 tests, all bottles were gently shaken. This comes with additional energy input and
571 maintenance costs which should be considered in the bigger economic picture of
572 TAN recovery. Contact between urine and bacteria could also be achieved via non-
573 mechanical means such as a plugflow through a tubular hydrolysis reactor. Clogging
574 due to scaling could be prevented by carefully selecting reactor materials (Li et al.
575 2007). The main issue with urine instability would be the effluent quality and scaling
576 in downstream processes (e.g. membrane units), rather than scaling in pipes.
577 In this respect, a semi-decentralized hydrolysis tank might be considered, e.g. in an
578 apartment building, airport, or shopping mall, as it would allow more centralized
579 nutrient recovery. The scaling-resistant materials can prevent precipitation of residual
580 urine in the toilet and pipes (Li et al. 2007). However, as Ca^{2+} is the main scaling
581 agent and its removal rate and efficiency is improved with temperature, the closer to
582 the toilet the more residual heat can be used to precipitate CaCO_3 .
583 The accumulated precipitates in hydrolysis tanks in individual toilets or in semi-
584 decentralized systems should be periodically harvested (Gagnon 2016) and could be
585 used as a household fertilizer, since urine-micropollutants don not accumulate in the
586 precipitates (Ronteltap et al. 2007). On a household level, 4 g d^{-1} of urine-derived
587 precipitate (i.e., struvite, CaCO_3 , and all phosphates) could be harvested. An easily
588 replaceable conical cartridge at the bottom of the toilet that can be collected at the
589 same time as other household waste could allow user-friendly precipitate harvesting
590 (Randall and Naidoo 2018). It can then be centrally processed in a safe manner and
591 sold as fertilizer after quality assurance. Overall, urine hydrolysis in the toilet provides
592 the advantage of optimal downstream nutrient recovery through scaling prevention
593 and NH_3 release and preservation.

594

595 **CONCLUSIONS**

596 As urine is unstable, nitrogen recovery from source-separated urine requires
597 controlled TAN release and divalent cation removal to avoid scaling in downstream
598 processes. This can be achieved by stabilizing urine at the toilet, without chemical-
599 input but as a controlled biological ureolysis as studied in this paper. To characterize
600 the best conditions for decentralized ureolysis and determine the essential
601 parameters for reactor design, ureolysis, divalent cation removal, and fermentation
602 were studied in anaerobic sequencing batch reactors.

- 603 - Ureolysis, calcium precipitation, and fermentation improved at 28°C compared
604 to 15°C indicating urine hydrolysis should occur in or close to the toilet.
- 605 - Autofermented urine as inoculum improved Ca^{2+} and Mg^{2+} precipitation rates
606 and efficiencies, while ureolysis was not impacted by the inoculum type.
- 607 - Specific activity rates were much higher in the batch activity test compared to
608 the SBRs in steady state operation, due to harsh physicochemical conditions.
- 609 - Struvite and calcite were the main crystals precipitating.
- 610 - Ureolysis was the only process significantly impacting the microbial community
611 composition.

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