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Inactivation of the urease enzyme by heat and alkaline pH treatment

Retaining urea-nitrogen in urine for fertilizer use

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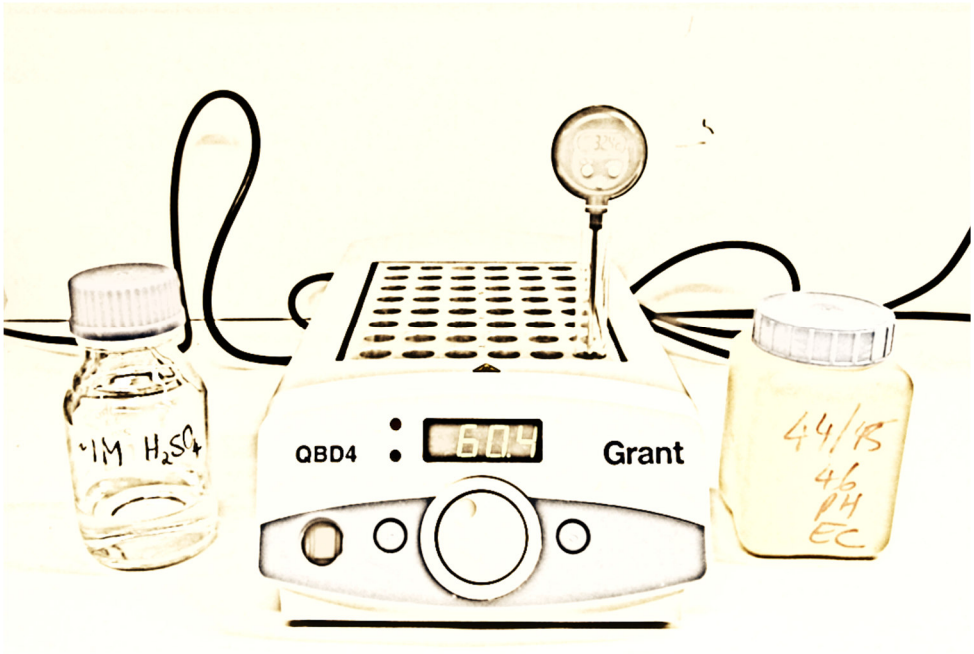

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Master thesis 30 HP

Magdalena Geinzer

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ABSTRACT

Urine, containing nitrogen (N) in the valuable form of urea, has a high fertilizer potential if it is dehydrated and the nutrients are concentrated. However, urea is not only a valuable N source for plants but also for microbiota which possess a highly efficient urease enzyme to degrade urea into volatile ammonia (NH_3). Since cross-contamination with microbiota, e.g. by faeces, cannot be 100 % prevented, it was intended to find feasible options to inactivate the urease enzyme and retain urea as N fertilizer in the urine. Therefore, faecal-based urease was examined regarding its sensitivity towards heat and alkaline pH as single and combined factors and depending on time of exposure. Potential approaches have been found to retain urea in human urine with the aim to concentrate the nutrients in the urine by dehydration to use it as a valuable fertilizer for plants. The results of this study emphasize the need to inactivate the urease enzyme in urine-drying systems and proved the high stability of urease at high temperatures. Although this study confirmed that urease show a high activity at 60 °C, has a high stability at high temperatures of ≥ 80 °C and partly regains its activity after being exposed to pH 12, efficient treatment options for urease inactivation were found. Particularly, the inexistence of any urea hydrolysation after an exposure to a pH of 13 and the high success of urease inactivation in the combined treatments of heat and alkaline pH give a direction towards new system options.

Keywords: Fertilizer, Nutrient recycling, Source separated Urine, Urea, Urease, Urine drying

POPULAR SUMMARY

There are two ways to look at the amount of plant nutrients in the environment: There can either be too little or too many. On the one side, farmers need to add nutrients in the form of fertiliser to grow food. On the other side, wastewater treatment plants try to remove as much nutrients from the household wastewater as they can in order to prevent pollution of the environment by too many nutrients (eutrophication). Urine is the part of the household wastewater which contains the major fraction of nutrients but much less pathogens or heavy metals than faeces or the rest of the household wastewater, e.g. what comes from sinks and washing machines (greywater).

Considering this, it means every time someone uses the toilet, valuable nutrients are flushed down into the sanitation system and are lost from fertilizer use. Thus, collecting and processing urine separately from the household wastewater could help to close the nutrient loop and obtain a natural fertilizer to grow food. Urine has one more advantage: Most of its nitrogen (N) is in the form of urea which globally accounts for more than half of all N fertilizer applied onto fields.

The major limitation for the use of urine is its high fraction of water (> 95 %). To decrease logistical problems for urine storage of approx. 550 L of urine per person per year (Vinnerås *et al.* , 2006) and costs for transportation and application onto the field, the nutrients in urine need to be more concentrated, e.g. by urine-drying.

However, there is a danger of losing this valuable urea since it is not only a valuable N source for plants but also for microbiota. Contamination, e.g. with faeces, cannot completely be avoided in urine-separating systems and microbiota can get into the pipes and urine storage tanks. A broad range of them possess an enzyme called urease which is able to degrade urea into ammonia (NH_3). NH_3 is volatile and can be lost during the drying process. Therefore, it is necessary to establish an inactivation treatment for urease before the urine is further processed.

Generally, enzyme activity is very susceptible to high temperatures as well as low (acidic) and high (alkaline) pH. This study intended to examine the sensitivity of urease from human faeces towards temperature and alkaline pH and investigate the possibility to inactivate the urease enzyme. This will help to retain N in the form of urea and obtain a high value N fertilizer.

Therefore, the sensitivity towards heat and alkaline pH of urease enzymes from faeces was examined. Further, it was tested if a longer exposure to the heat or alkaline treatments resulted in a lower urease activity. This study proved that urease has a high activity at 60 °C and it was not completely inactivated until after an exposure for 3 hours to 60°C or an exposure to high temperatures of > 80 °C. Urease was inactive as long as it was exposed to any pH above 10 but regained its activity after the treatment. Only an exposure to a pH as high as 12 partly inactivated the urease enzyme. Finally, a pH of 13 completely inactivated urease enzymes after the treatment – which is high considering that a normal cleaning soap has a pH of around 9. Interestingly, when urease was exposed to a combination of 60 °C and pH of 10, only 40 % of urea-N hydrolysation was left after only half an hour of exposure.

The results of this study will help to find practicable approaches to turn urine into a valuable, concentrated N fertilizer, close the nutrient loop and decrease the load on the wastewater treatment plants.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

$CO(NH_2)_2$	Urea
CO_2	Carbon dioxide
g/L	Gram per litre
H_2O	Water
H_2SO_4	Sulfuric acid
HCO_3^-	Hydrogen carbonate
K	Potassium
K_2O	Potassium oxide
KOH	Potassium Hydroxide
L	Litre
M	Mol
mg/L	Milligram per litre
Mt	Megaton
mV	Millivolt
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide
$NaCl^-$	Sodium chloride
NaOH	Sodium hydroxide
NASA	The National Aeronautics and Space Administration
NH_3	Ammonia
NH_4^+	Ammonium ion
Ni^{2+}	Nickel ion
N-tot	Total amount of nitrogen
OH^-	Hydroxide ion
Osc	Oscillations
P	Phosphorus
P_2O_2	Diphosphorus dioxide
pK_a	Acid dissociation constant at logarithmic scale
STDEV	Standard deviation
WWTP	Wastewater treatment plant

INTRODUCTION

Whereas N is in the top of the list of eutrophying pollutants and wastewater treatment plants (WWTPs) have a hard time to reduce its release in the effluent; it is a valuable plant fertilizer and produced costly in the highly energy demanding Haber-Bosch process.

In many cases the recovery of nutrients is energetically more efficient than removal in WWTPs and new-production of fertilizer (Maurer *et al.*, 2003). And yet, the conventional sanitation system is designed for the removal of nutrients, emitting them to the atmosphere and increasing greenhouse gas emissions (GHG), while at the same time investing large amount of operational energy for the removal process (Kampschreur *et al.* , 2009; Foley *et al.* , 2010). Considering this, the recovery of nutrients from wastewater suggests itself.

Since urine, compared to faeces and greywater, contains the major fraction of nutrients but much less pathogens or heavy metals, source-separated urine could help to close the nutrient loop, avoid highly complex removal processes of N and the eutrophication loads from WWTPs (Lundin *et al.* , 2000; Foley , 2010). Besides the environmental impact, human urine has one more advantage: The majority of its N is in the form of urea, the form in which globally more than half of all N fertilizer applied (Yara , 2011).

The major limitation for the use of urine is its large fraction of water (> 95 %) and thus a low concentration of N (0.6 %) compared to a commercial urea fertiliser (46 %). To decrease logistical problems for urine storage (approx. 550 L of urine per person per year (Vinnerås , 2006)) and costs for transportation and application onto the field, the nutrients in urine need to be concentrated. However, there is a danger to lose the valuable urea during this process since urea is not only a good N source for plants but also for microbiota. Due to faecal cross contamination, a biofilm of faecal microbiota develops in pipes and storage tanks (Vinnerås et al. 2007; Hotta et al. 2008).These microbiota release an urease enzyme which is highly efficient in the degradation of urea into NH_3 (Callahan *et al.* , 2005). NH_3, in contrast to urea, is volatile and can easily be lost to the atmosphere. Therefore, it is necessary to establish an inactivation treatment for urease before storage and further processing of the urine.

The idea of urine drying is not new and several approaches were studied. Already in 1971, Putnam (1971) investigated the possibility of water evaporation in urine for the NASA and according to Maurer et al. (2006) it is one of the most effective volume reduction treatments. High temperatures are a major technique to increase the drying rate, together with a good drying media such as ash or biochar (Dutta , 2012; Simha , 2016). The drying medium provides a larger surface area for moisture removal and break down urine peptide films which form during dehydration preventing efficient evaporation.

Generally, enzyme activity is very susceptible to high temperatures as well as acidic and alkaline pH. However, there is a lack of knowledge about the effect of pH and temperature on the activity of urease from faecal sources and the irreversibility of this inactivation. The irreversible inactivation of urease would allow a broader flexibility in technologies for urine drying, including pipe transport and storage of urine when second contamination is inhibited.

OBJECTIVES

This study intended to examine the sensitivity of faecal-based urease towards temperature and pH. The overall objective of this study was to identify optimal urine treatment options to effectively inactivate urease enzymes and retain urea as nitrogen fertilizer in the urine. The following three research questions were answered:

- How much time is required to inactivate urease at temperatures of 60, 80 and 95 °C?
- How much time is required to inactivate urease at pH values of 10, 11, 12 and 13?

And most of all:

- Is this inactivation reversible?

LITERATURE

The contradiction of Nitrogen: Economical value and environmental pollution

On the one side, reactive N is an economically valuable fertilizer, costly produced in the Haber-Bosch process, on the other side it is seen as an environmental pollutant, leading to eutrophication and global warming.

Valuable fertilizer

According to the International Fertilizer Association (IFA), 172.2 Mt of N + P_2O_2 + K_2O were used globally in 2010, out of which 104.3 Mt account for N fertilizer alone (Heffer , 2013). Whereas since 1960, the use of N fertilizer has been increasing, prices for mineral fertilizer has been increasing as well (USDA 2013). This is due to the increasing demand and the high energy consumption for the Haber-Bosch process which is the major technique of N fixation (Galloway *et al.* , 2008).

Nitrogen as environmental pollutant and greenhouse gas

The problem of eutrophication and environmental pollution by WWTPs was already addressed by the European Council in 1991. Two legislations were adopted: The Nitrate Directive (91/676/EEC) and the Urban Wastewater Treatment Directive (91/271/EEC) (Council of the European Communities 1991a; 1991b). In the year 2000, the water framework directive was adopted "to avoid long-term deterioration of freshwater quality and quantity" (European Parliament and the Council of the European Union , 2000). Although WWTPs decreased N in their effluent in order to fulfil the EU regulations, they are still a source for environmental pollution: For example, in 2013, 75 434 t nitrogen were discharged from the 9600 WWTPs in Germany which measured the amount of tot-N in the effluent (another 247 WWTPs did not measure the N discharge in their effluent) (Statistisches Bundesamt , 2015). Further, by the enhanced developed techniques to decrease N in the effluent, Dinitrogen (N_2) and Nitrous oxide (N_2O) is emitted from wastewater treatment processes into the atmosphere (Tallec *et al.* , 2006; Kampschreur , 2009). N released in the form of N_2O into the atmosphere is a potent greenhouse gas.

Although sanitation and clean water is one of the Sustainable Development Goals (SDGs), 2.5 billion people still lack access to sanitation services (UN News Centre , 2016). The majority of those people's livelihood is dependent on agricultural production. Local farmers rarely can afford the use of mineral fertilizers due to their increasing prices, which leads to a decrease in production worsening poverty and hunger (Sanchez , 2002).

Subsequently, there is an urgent need to recycle reactive N from households as an economically valuable nutrient in a closed loop recycling economy.

Sanitation systems and nitrogen recycling

There exists a variety of sanitation systems and the most feasible option depends on factors such as access to central WWTPs (Berndtsson , 2006), local environment (temperature, rainfall, soil), culture, resources, capital or maintenance costs (Tilley *et al.* , 2008) and the focus of the system such as health or environmental impacts (Langergraber and Muellegger , 2005).

On the one hand, sanitation can be centralized at a central WWTP. On the other hand, many localized sanitation approaches exist (Tilley , 2008). Further, there are different collection strategies for nutrients from households but they differ in the fraction of collected nutrients (Vinnerås , 2002). The conventional centralized system collects all nutrients from urine and faeces. However, the collected fraction is pooled

with flushed water from the toilet and often mixed with other collected conventional household wastewater (i.e. with greywater from sinks and washing machines) which can add higher levels of heavy metals and other contaminants than pure urine. Most of the heavy metals are derived from detergents and dust in households (cadmium) or anti-fungal (dandruff) shampoo (zinc). A blackwater system aims to collect only urine and faeces and is not mixed with greywater from sinks and washing machines but the collected fraction is diluted with flushed water and can be contaminated with cleaning water from the toilet (Vinnerås , 2002).

Human excreta can also be a source of heavy metals. Heavy metals are of minor biological usage in the body. Thus, their up-take into the body is regulated and they are mostly excreted in the faeces whereas pure urine contains little heavy metals (Figure 1).

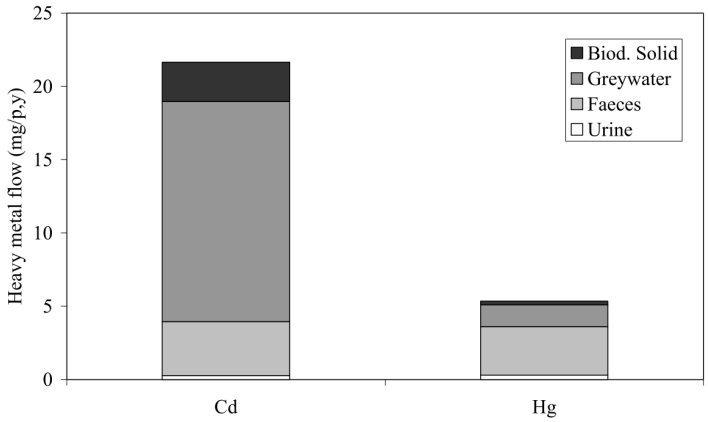

Figure 1. An example of the amount of the heavy metals in the fractions of wastewater and biodegradable solid waste: Cadmium and mercury (Vinnerås , 2002).

On the contrary, urine contains the majority of the excreted nutrients. Thus, in urine-separating toilets a concentrated fraction could be obtained to be used by urine-drying systems while at the same time heavy metals and other contaminants from greywater and other sources could be avoided (Vinnerås , 2002). This nutrient recycling can reduce the emission of N_2O, reduce the load of WWTPs and costs as particularly N removal processes within the WWTP are complex and require large operational costs (Lundin et al. 2000, Kampschreur et al. 2009). According to Jönsson et al. (1997), 80 – 100 % of urine can be correctly separated in urine-separating toilets. The average contamination with faeces is low with approximately 1-9 mg faecal material/L urine solution which is equivalent to approximately 10^{-6} - 10^{-5} (Jönsson , 1997; Schönning *et al.* , 2002).

Human urine: chemical composition and Nitrogen fertilizer potential

Great financial benefits of using urine as fertilizer compared to artificial fertilizer are shown by Berndtsson (2006) including, additional to the mentioned reduced loads to the WWTP, reduced costs for water use in dry urine-separating systems and the value of nutrients in separated urine.

A survey in Sweden by Berndtsson (2006) reported that farmers generally show a positive attitude towards the use of human urine in agriculture and state that it is “generally felt to be natural”. In the consumer survey within the same study, 77 % of the participants expressed a positive attitude towards the collection of human urine for the use as a fertiliser. Another survey confirmed this positive attitude by the majority of the farmers in South India (Simha *et al.* , 2017). Although cultural aspects have to be considered and a study in Ghana found a generally negative attitude towards using human excreta (Mariwah and Drangert , 2010), more studies report a positive attitude of farmers and consumers e.g. in Switzerland (Lienert *et al.* , 2003; Pahl-Wostl *et al.* , 2003).

Compared to conventional fertilizers, urine is very diluted and consists of around 95 % water (Kirchmann and Pettersson , 1994). The Swedish design value of urine wet mass proposed by Vinnerås et al. (2006), is 555 kg per person per year. Whereas most of the excreted organic carbon is contained in the faeces, urine contains most excreted nutrients. Regarding N that means 80 – 90 % of 4.6 kg excreted N per person annually contained in the urine (Vinnerås , 2006). Undiluted fresh human urine contains between 7-9 g N per L in total, of which around 85 % is present as urea. Other N compounds are NH_3, uric acid and amino acid-N (Kirchmann , 1994).

Compared to the average conventional fertiliser applied in Sweden or applied sewage sludge from WWTPs, urine from separating toilets contains favourable less heavy metals (Jönsson , 1997) and thus application of urine will not increase heavy metals in soils according to agronomic practices (Kirchmann , 1994). Of other excreted fertilizer compounds, urine contains 50-80 % of 0.55 kg excreted P and 80-90 % of 1.2 kg excreted K per person per year (Vinnerås , 2006). The large content of N results in a lower P/N and K/N ratio than many mineral fertilizer (Jönsson *et al.* , 2004). The exact amount of all compounds in urine highly depends on the personal diet (Hernandez *et al.* , 1987).

The high urea content is beneficial since of worldwide around 100 million tons of N fertilizer yearly, 56 % is applied in the form of urea (Yara , 2011).

Due to the large content of water of > 95 %, (Randall *et al.* , 2016), the nutrient concentration per L is low. The low nutrient concentration requires application of large quantities of urine per hectare, creates logistical problems for urine storage (approx. 550 L of urine per person per year (Vinnerås , 2006)) and increases costs for transportation to and application onto the field. Thus, for an efficient use of urine as fertilizer, urine-drying to concentrate the nutrients in the urine can be advantageous. This would allow an easy transport of urine to, and application of a highly-concentrated fertiliser onto, the field.

Urea degradation

Spontaneous urea hydrolysis is very slow compared to many other biological reactions, with a half-life of 40 years at room temperature and neutral pH (Table 1). The half-life decreases with increasing temperature and slightly with increasing pH, however, urease enzyme is very efficient in enhancing the rate of this reaction.

Table 1. Examples of the half-life of urea varying on temperature, pH and catalyst (Senecal & Vinnerås 2017)

	t½
Unanalysed (neutral pH, 25 °C)[a]	40 years
20 °C (pH > 10)[b]	No hydrolyses measured over 32 days
38 °C (pH < 12)[c]	3.6 years
65 °C (pH < 12)[d]	15.3 days
65 °C (pH > 12.5)[d]	14.1 days
Enzymatic (Jack-bean, neutral pH, 25 °C)[a]	0.02 s

a (Callahan , 2005)
b (Kabdaşlı *et al.* , 2006)
c (Zerner , 1991)
d (Warner , 1942)

Urine dry matter is often biochemically decomposed during pipe transport and storage, decreasing to one-third of its original dry mass (Vinnerås , 2006). Particularly the main compound, urea, is degraded to NH_4^+, hydrogen carbonate and hydroxide ion (Equation 1). This degradation is catalysed by the enzyme urease

(Callahan , 2005) which is present in many microorganism as well as in form of free urease enzyme as it is also produced as an extracellular enzyme. This makes it hard to prevent within urine collection and treatment systems (Alexander , 1977). Hotta et al. (2008) reported that with an amount of 10 g of faeces / L urine at 30 °C, the amount of NH_3-N increased almost by a factor of 10 within 5 days, compared to urine without contamination.

During the hydrolyses, NH_4^+, hydrogen carbonate and hydroxide ion are produced and the pH increases (Hellström *et al.* , 1999):

$$CO(NH_2)_2 + 3\,H_2O \rightarrow 2\,NH_4^+ + HCO_3^- + OH^- \qquad \text{(Equation 1)}$$

Ammonium and dissolved NH_3 are in equilibrium. The pK_a value for the equilibrium between NH_4^+ and NH_3 is 9.3 at 25 °C (Snoeyink and Jenkins , 1980, p. 447):

$$NH_4^+ + OH^- \leftrightarrow NH_3\,(aq) + H_2O \qquad \text{(Equation 2)}$$

Further, there is an equilibrium between dissolved NH_3 in the liquid and gaseous NH_3 above the liquid surface showing the importance of the partial pressure of NH_3 to retain NH_3 in the liquid (here urine):

$$NH_3\,(aq) \leftrightarrow NH_3\,(g) \qquad \text{(Equation 3)}$$

Due to the production of OH^-, the pH in urine increases to slightly alkaline. This favours the decomposition of uric acid, which is insoluble at lower pH, to urea and from there further to NH_3.

A higher pH shifts the balance from NH_4^+ to NH_3 (Equation 2), which can be lost by volatilisation (Equation 3). Low utilization rates of NH_3 in urine for plants were shown due to high gaseous losses (Kirchmann , 1994). The large amount of hydrogen carbonate and NH_3 leads to a high buffer capacity of stored urine at around pH 9 (Udert *et al.* , 2006).

High losses of nitrogen in separating systems can be reduced by a decreased gas exchange rate for example decreasing ventilation flow by creating a closed system or decreasing the surface area (Pereira and Trindade , 2014).

Even in urine-separating toilets, urease accumulates in urine piping systems and toilet bowls due to cross contamination from faeces (Vinnerås , 2002). Urease activity and non-enzymatic urea hydrolysation increase the pH and lead to a precipitation of phosphate, magnesium, calcium and NH_4^+ and the build-up of mainly struvite, apatite and calcite (Udert et al. 2003). This build-up serves as an anchor for many urease-producing bacteria with high urease activity (Oki *et al.* , 2010). In an open system, this will lead to high losses of volatile NH_3 (Equation 3). Thus, losses need to be prevented by the inactivation of the urease enzyme.

Urease

Urease catalyses the reaction of water with urea which results in two NH_3 molecules and one hydrogen carbonate. Although the general structure of the urease enzyme can vary, the structural biology of the active site of all bacterial urease is similar containing two Ni^{2+} as prosthetic group (Figure 4) (Benini , 2013, p. 2287). Increasing the decay by 10^{15} times in neutral solutions at 25°C, the efficiency of the urease enzyme is even slightly higher than the efficiency of other metallo-enzymes that catalyse hydrolysis of similar bonds (Callahan , 2005).

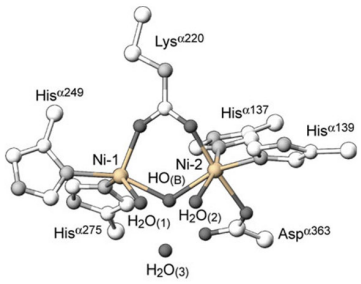

Figure 2. Urease, coordination geometry of the two Ni^{2+} ions in a native urease active site (Benini , 2013).

There are many different types of intra- as well as extracellular urease in the environment, in pathogenesis and in the gastrointestinal tract (Mobley and Hausinger , 1989). The gastrointestinal tract of humans represents a large microbial ecosystem with several trillion microbial cells (Lynch and Pedersen , 2016). In a study from 1979 by Suzuki et al. (1979; Figure 5), 120 strains of bacteria were isolated from human faeces in which *E. aerofaciens*, *E. lentum* and *P. productus* were the predominant ones. Those strains were reported to have a particular high urease activity.

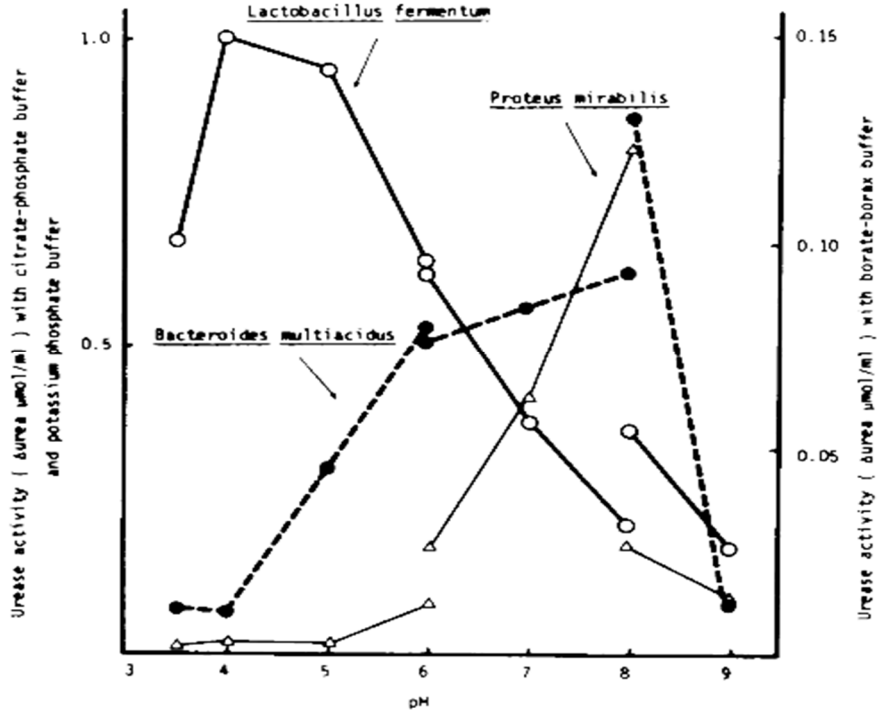

Figure 3. Different pH activity curves of the urea-hydrolyses of intestinal bacteria (Suzuki et al. (1979). For determination of the urease activity, the following buffers were used: 0.2 M citrate-phosphate buffer at pH below 6.0, potassium phosphate buffer at pH 6.0 to 8.0, and borate-borax buffer at pH above 8.0.

Nevertheless, Krajewska et al. (2012) reported that the active site of the urease enzyme from many different sources was found to be almost completely superimposable. According to Krajewska et al. (2012), this allows the generalization of experimental data from one urease enzyme to other urease enzymes.

Liu et al. (2008) demonstrated in an experiment about the urea hydrolyses by microbial urease at high pH (>9) that urease activity decreased significantly at a high pH. According to this study, this was caused by a faster dying rate of urea hydrolysing bacteria at a higher pH compared to a lower pH of 6.

Another study in which pure jack bean urease enzymes were added to untreated human urine showed that no urea hydrolyses occurred at a pH higher than 10 (Kabdaşlı et al. 2006).

According to Qin & Cabral (1994), the hydrolyses of urea (Michaelis-Menten constant) of jack bean urease remained almost constant during a pH of 6.5 – 8, with the maximum activity of urease being at 7.2 pH. Further, only slight changes could be detected at temperatures between 20 – 40°C. The urease activity decreased with the addition of buffers and salts and this effect increased significantly with an increased pH so that a combination of a high ion strength (0.1 M) and pH (> 8.5) inhibited urease activity (Qin , 1994). Hotta & Funamizu (2008) proved those results for urease obtained from human faeces. In their study, they determined that a high concentration of NaCl (150 g/l) at neutral pH was required to obtain a urea hydrolysation rate reduction of 50 %. Human urine has an average NaCl value of 8 g/L (Putnam , 1971).

Further, the enzyme activity is highly temperature dependant. In the study by Liu et al. (2008), the highest NH_3-N value was reached at 30 °C after 36 h, whereas at temperatures of 2.5 and 10 °C only < 40 % of this high value was hydrolysed.

Eremev (1999) studied the activity of jack bean urease at temperatures between 20 and 50 °C, with varying pH. Their results showed the lowest activity of jack bean urease at 20 °C and the highest activity at 50 °C (Figure 4) with an optimal pH between 4 and 7.

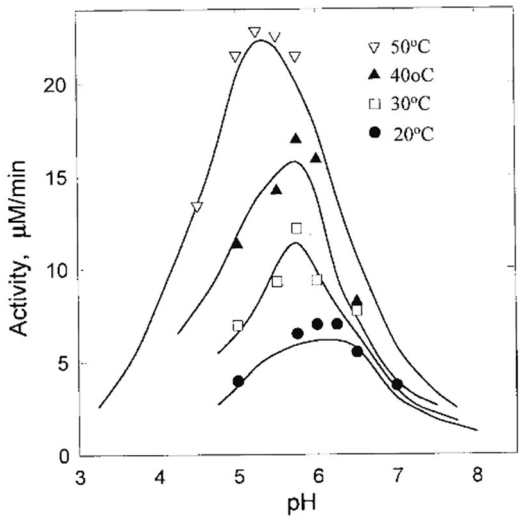

Figure 4. Enzyme activity is temperature and pH depending (Eremev , 1999).

The half-life of jack bean urease was reported to be around 110 h at a pH of 7, a temperature of 25 °C and an urea concentration of 1 mg/L (Qin , 1994). In several trials, Hotta (Hotta & Funamizu 2008, Hotta et al. 2008) reported that faecal urease activity completely terminated after several days even though urea nitrogen remained. Thus, the authors concluded that urease activity was not eliminated but inhibited either by the high pH (~ 9) or concentration of free NH_3 nitrogen.

Krajewska et al. (2007) determined an optimum temperature for jack bean urease of 65 °C at a pH of 7 in a phosphate buffer system. However, denaturation of the enzyme was reported. At a temperature of 70 °C the half-life for urease was 120 min but 40 % activity was still measured after 250 min. At a low temperature of 4 °C the activity was retained for 8 days but decreased rapidly thereafter, whereas at 25 °C, the half-life of urease activity was 3 days (72 h) which was less than the reported half-life in the buffer-free system by Qin & Cabral (1994) (110 h).

Another study reported that, although the highest activity could be measured at 65 °C, after 60 min all enzyme activity of jack bean urease was lost. The determined half-life at this temperature was 19 min (Arica , 2000).

Little literature is available about the half-life of faecal urease. Further, there is a lack of literature about the reversibility of the effects of high pH and temperatures on urease. Irreversible inactivation occurs by irreversible denaturation. At high temperature or pH, the native structure of the enzyme is disrupted and non-native bonds might be formed instead. When the temperature or pH increases above a certain level, non-

native bonds can be retained after the exposure due to kinetic reasons as the mobility of polypeptide chains decreases (Martinek *et al.* , 1980). The loss of prosthetic groups, the dissociation into subunits or conformational changes (alteration of the tertiary structure of an enzyme) may lead to irreversible structural alterations (Mozhaev and Martinek , 1982). According to a study about the thermal irreversible inactivation of urease by Illeová et al. (2003), the native form of urease first reversibly dissociates into denatured forms. Those denatured forms associate irreversibly with either the denatured or the native forms.

Techniques for N recovery

A large number of processes are available to recover N from separated urine. However, all processes have their advantages and disadvantages and differ in N yield. Whereas in reverse osmosis or ion exchange with zeolite only 60-80 % N recovery is achieved; in evaporation, electro dialysis and struvite precipitation (with stoichiometric phosphate addition) 90 % recovery can be obtained (Maurer , 2006). Nevertheless, the implementation of those treatments requires either a high chemical input or the system is very complex. Udert et al. (2006) stated that acidification of stored urine to prevent a large NH_3 volatilisation is not economical due to the high buffer capacity of urine. Maurer et al. (2006) stated that "considerable development work remains to be done in order to enhance urine-processing techniques into marketable products". In an experiment by Dutta (2012), it was shown that more urea could be retained at a highly alkaline pH above pH 10 compared to lower pH values.

An irreversible inactivation of urease would allow the subsequent water removal of urine and the application of urine as concentrated fertilizer on the fields without creation of strong smell (Randall , 2016).

This study aimed to examine if an inactivation of urease at temperatures of 60, 80 and 95 °C or pH values of 10, 11, 12 and 13 after different exposure times is irreversible and how much urea is degraded during the treatment.

MATERIALS

To determine the effect of pH and heat on urea hydrolysing activity of urease in urine, three experiments were set up to examine the effect of the single factors and their combined effect. Therefore, human urine and faeces, containing bacteria which produce the urease, were collected.

Urine

Urine was collected during excretion in sterile 500 ml plastic bottles from random people at the department and housing partners of the colleagues. The bottles were stored at 4 °C until use, to a maximum of 2 months for Experiment 1 and a storage time of maximum 2 weeks for Experiment 2 and 3. Two random controls with fresh urine showed no significant different behaviour between stored and fresh urine. Contaminated urine bottles which were recognized by an unclear liquid, precipitates or a pH above 7 were discarded. For the preparation of the urine samples, urine from three different, randomly chosen bottles was pooled and a sample was taken from the mixed urine.

The amount of urea in the pooled urine was randomly measured twice (with duplicates) during the experiment using jack bean urease for urea hydrolysation. For the calculation of how much jack bean urease was required, 10 g/L of urea was assumed in the urine. Per g of urea, 5000 U urease enzymes were added which accounted for 50 mg corresponding to 250 U per test tube of 5 ml (Senecal and Vinnerås , 2017):

$$\frac{0.005\ L}{1\ L} * 10\frac{g}{L} * 5000\ U = 250\ U$$

Since 1 mg of urease contained 5 U urease enzymes, 50 mg urease were added per tube (5 ml):

$$1\ mg \times \frac{250\ U}{5\ U} = 50\ mg$$

Faeces

Fresh faeces, no older than one day, were collected in plastic bags, normally used for collection of dog faeces. Faeces from three different donors were used and well mixed by manually kneading inside a plastic bag until a uniform mass was produced and at least for 3 minutes. A faeces solution with a ratio of 2:1, 2 parts deionized water and one part faeces, was prepared to simplify the later application of faeces to the sample. It was vortexed until all dispersible parts were dissolved and a uniform solution was obtained with a minimum time of 3 minutes. To allow a uniform distribution of the urease within the solution, the faeces solution was either prepared a day before and kept in the fridge overnight or shaken in a shaker at 200 osc/minute for at least 0.5 hours.

Apparatus and chemicals

All apparatus and chemicals used in the described experiments are listed in table 2.

Table 2. Instruments and chemicals used during the project

Apparatus	Specification	Producer
Ammonium chloride NH_4Cl	0.1 M, 0.01 M, 0.001 M	SVA, Sweden
Beaker	50 ml	
Buffer solutions	4, 7, 10, 12.46 CertiPUR	Merck, Germany
Electronic stirring table	Big-squid	IKA, Germany
Faeces bags	Generic dog pooh bags	
Heat block	QBD4	Grant Instruments, UK
pH and NH_3 meter	pHM210 standard pH meter	Radiometer Copenhagen, Denmark
	pH electrode BlueLine 14 pH 0-14/ -5-100 °C/	
	NH_3 electrode, 6.0506.150, 0-50 °C	Metrohm AG, Switzerland
Pipettes	0.1 – 25 ml	Eppendorf, Germany
Scale	Adventurer Pro Digital scale, g with 2 decimal	Ohaus Corp., USA
Shaker table	Rotamax 120	Heidolph, Germany
Jack bean urease	Lyophilised, 5 U/ mg	Merck, Germany
Sodium hydroxide (NaOH)	10 M, 5 M, 1 M	SVA, Sweden
SPSS Statistics	Version 20	IBM, USA
Sulphuric acid (H_2SO_4)	1 M, 0.1 M	Fisher Scientific, USA
Test tubes	15 ml	Eppendorf, Germany
Thermometer	Fluke 52 K/J	John Fluke MFG.CO.INC, USA
Urine bottle	500 ml, sterile, HDPE with separate seal	Gosselin, France
Volumetric flask	100 ml	Eppendorf, Germany
Vortex	Analog mini vortex mixer	VWR, Sweden

METHODS

The same methods for measuring the amount of hydrolysed urea-N and the pH were used throughout the experiments and are explained once in the following section. All samples were destructive samples, thus destroyed after each measurement.

Experiment 1: Heat treatment

Three different temperatures were tested for the ability to inactivate urease: 60, 80 °C for 1 and 3 hours and 95 °C for 0.25, 0.5, 1 and 3 hours. Three repetitions and two controls were processed for each temperature to include the aspect of variation in urine and faeces properties like amount of NH_3, pH or urease activity. One control was a urine-faeces mix at room temperature (22 °C) (henceforth called UF) and one control was conducted without faeces, U60, U80 and U95 respectively (Table 3). Each repetition was produced on different days with different urine and faeces samples to make sure that the samples were randomized. This allowed a comparison of the results from different treatments where different urine and faeces mixtures were used. Due to reasons of time and schedule, the treatment UF_T60 was conducted on different days than UF_T80 and UF_T95, thus different faeces and urine samples were used which lead to a different amount of urea-N hydrolysation in the respective control.

Table 3. Summary of the labels of all treatments in Experiment 1

Label	Constituent	Temperature	Sample times
U_T60	Urine	60	1 and 3 hours
U_T80	Urine	80	1 and 3 hours
U_T95	Urine	95	0.25, 0.5, 1 and 3 hours
UF[1]	Urine + faeces	22 (room temperature)	0.25, 0.5, 1 and 3 hours
UF_T60	Urine + faeces	60	1 and 3 hours
UF_T80	Urine + faeces	80	1 and 3 hours
UF_T95	Urine + faeces	95	1 and 3 hours

[1] UF for UF_T60 was conducted with different urine and faeces samples than UF for UF_T80 and UF_T95.

Procedure of treatment

For the testing, 5 ml of mixed urine was pipetted into the 15 ml test tubes and 0.30 – 0.35 g of the faeces solution was added, corresponding to 0.1 g of pure faeces. After the addition of the faeces solution, the test tubes were closed and vortexed for two seconds.

Then test tubes were directly put into the heat block. Water was added to the heat block beforehand to accelerate the heat transfer to the test tubes and the heat block was pre-heated to the respective temperatures. After treatment exposure time, the test tubes of the samples were taken out of the heat block and cooled down to room temperature in water of room temperature before the measurement of pH and NH_3-N. To examine the re-activation, second test tubes with the same treatment were taken out at the same time, cooled down, and stored at room temperature. After a total of 24 hours, including heating and cooling, the pH and NH_3-N content of those samples was determined. Figure 5 illustrates the treatment procedure.

In order to determine the inactivation of the urease, the amount of NH_3 was measured representing the amount of hydrolysed urea-N. The pH and NH_3 content was measured as follows:

The pH and NH_3 at time = 0 was measured as a control. The test tubes were kept close and were only opened once the test tube felt to have reached room temperature by skin contact, then the exact temperature was determined with a thermometer.

The pH was measured as soon as the samples reached room temperature. The pH electrode was flushed with deionized water after each sample.

After the pH measurement, the 5 ml samples were diluted with 45 ml of deionized water and 0.5 ml of 10M NaOH was added for the measurement of NH_3-N with an NH_3 electrode.

For the NH_3-N measurement, a calibration curve was prepared to allow the calculation of the NH_3-N content in the sample according to the voltage, given by the device: for the calibration curve, 0.5 ml of NaOH was added to 50 ml of 0.001, 0.01 and 0.1 M ammonium chloride.

Then a conversion of the given mV into mg of NH_3-N per L was calculated according to the equation given by the calibration curve and the molar mass of N (14.001 g/L), considering the dilution factor (10) and converted to mg/L.

The resulting voltage highly depends on temperature and speed of stirring, thus the calibration was verified every day and the equation adjusted. The same speed of stirring was used for all measurements. In between the measurements, the electrode was flushed in deionized water for at least 10 minutes and until the mV reached at least 80 % of its original value. The overall accuracy of the measurements, including the variance in addition of faeces solution, was considered to be around 10 %.

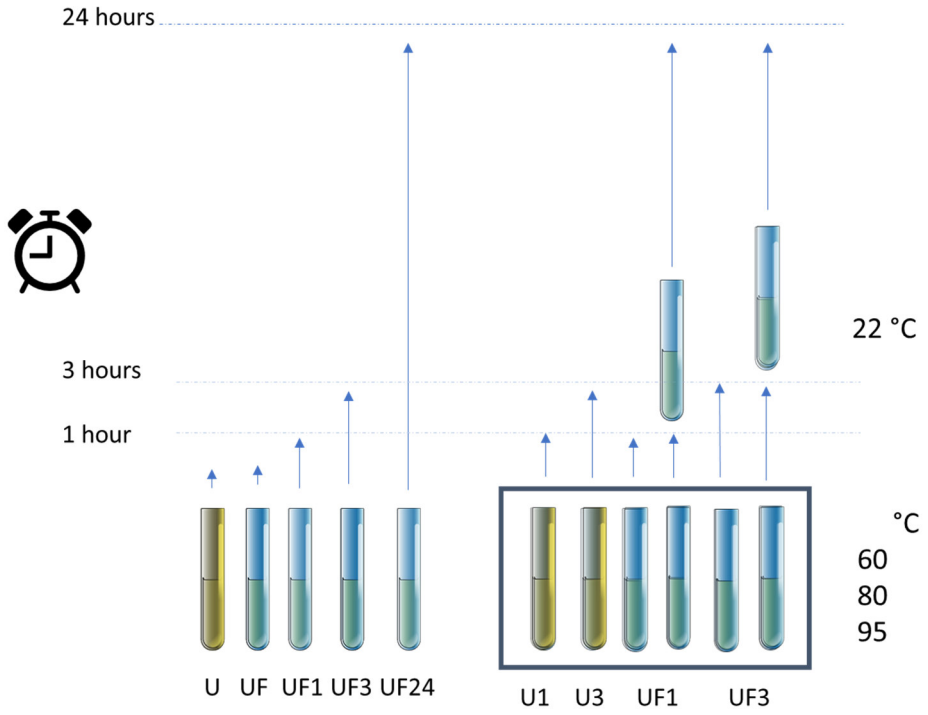

Figure 5. Treatment procedure of the heat treatment. Controls without heat treatment were measured at the start and after 0.25, 0.5, 1, 3 and 24 hours. For the treatment samples, two tubes were taken out of the heat block after the respective exposure time: one was measured directly, the other left at room temperature until a total of 24 hours was reached. U=Urine, UF = Urine + Faeces, cipher indicates the time of exposure.

Experiment 2: pH treatment

Four different pH values were tested for their ability to inactivate urease: pH of 10, 11, 12 and 13 (henceforth referred to as UF_pH10, UF_pH11, UF_pH12, UF_pH13, respectively) at room temperature (22 °C). Three repetitions for each of the pH values were processed on different days with different urine and faeces samples. Two control treatments were added: One without pH manipulation (UF) and one without addition of faeces solution (U_pH10, U_pH11, U_pH12, U_pH13) (Table 4).

Table 4. Summary of the labels of all treatments in Experiment 2

Label	Constituents	pH	Sample times
U_pH10	Urine	10	1, 3 and 24 hours
U_pH11	Urine	11	1, 3 and 24 hours
U_pH12	Urine	12	1, 3 and 24 hours
U_pH13	Urine	13	1, 3 and 24 hours
UF	Urine + faeces	No manipulation	0.5, 1, 3 and 24 hours
UF_pH10	Urine + faeces	10	0.5, 1, 3 and 24 hours
UF_pH11	Urine + faeces	11	0.5, 1, 3 and 24 hours
UF_pH12	Urine + faeces	12	0.5, 1, 3 and 24 hours
UF_pH13	Urine + faeces	13	0.5, 1, 3 and 24 hours

Procedure of treatment

To adjust the pH in the samples and the urine control without faeces, 50 ml of urine were transferred into a 100-ml beaker and 5 or 10 M NaOH was added with a pipette (several drops – 5ml, depending on the required pH), while being mixed on the magnetic stirring table, until the required pH was reached. The added amount was noted down. Afterwards, 5 ml of the urine was transferred into single test tubes and 0.30 - 0.33 g faeces solution was added.

For the NH_3 measurement, the samples had to obtain the same dilution factor. Depending on the amount of added NaOH to the beaker, the sample was mixed with deionized water to reach a dilution factor of a total of 45:5 ratio ml diluting liquid to urine. For example, if 5 ml of NaOH were added, it was assumed that 0.5 ml were added to each sample:

$$\frac{5\ ml\ NaOH}{\frac{50\ ml\ urine}{5\ ml\ sample}} = 0.5\ ml\ NaOH$$

$$45\ ml - 0.5\ ml\ NaOH = 44.5\ ml\ deionized\ water$$

Thus, this sample was diluted with 44.5 ml of deionized water. The required amount of deionized water was calculated for each treatment to obtain an equal dilution. NH_3-N was measured as described in section Experiment 1: Heat treatment.

To determine the re-activation, the pH of second test tubes with samples was re-set to a pH value of 7.2 ± 0.2 at the same time. This was done by adding several drops up to 2 ml of 1 or 5 M H_2SO_4. The amount of added H_2SO_4 depended on the pH value of each sample. In some cases, the exact calibration to a pH of 7.2 required the use of 1 M NaOH and 0.1 M H_2SO_4.

After a total of 24 hours, the pH and NH_3-N content of those samples was measured following the Procedure of treatment as described in Experiment 1. To maintain the correct dilution factor in the NH_3-N measurement, sample tubes were weighed and the required amount of deionized water was adjusted to reach a total ratio of 45 ml to 5 ml urine. It was assumed that 1 g of NaOH or 0.1 M H_2SO_4 equals 1 ml.

Experiment 3: Combined treatment

It was examined if urease could be inactivated with a combined treatment of heat and pH. Therefore, a combined treatment of 60 °C and 3 different pH values, 10, 11 and 12, was conducted. Three repetitions for each of the treatments (UF_pH10_T60 … UF_pH12_T60) were processed and measured after 0.5, 1, 3 and 24 hours. Two controls were added: one control with faeces solution for 3 and 24 hours without any manipulation and at room temperature (22 °C) and one control (U_pH10_T60 – U_pH12_T60) without addition of faeces solution for each of the treatments (Table 5).

Table 5. Summary of the labels of all treatments in Experiment 3

Label	Constituents	pH & Temperature	Sample times
UF_pH10-T60	Urine + faeces	pH 10. 60 °C	0.5, 1, 3 and 24 hours
UF_pH11-T60	Urine + faeces	pH 11, 60 °C	0.5, 1, 3 and 24 hours
UF_pH12-T60	Urine + faeces	pH 12, 60 °C	0.5, 1, 3 and 24 hours
UF	Urine + faeces	No manipulation, room temperature (22 °C)	3 and 24 hours
U_pH10-T60	Urine	pH 10. 60 °C	3 and 24 hours
U_pH11-T60	Urine	pH 11, 60 °C	3 and 24 hours
U_pH12-T60	Urine	pH 12, 60 °C	3 and 24 hours

Procedure of treatment

To determine the pH and amount of NH_3-N, the same procedures as in Experiment 1 and 2 were followed using pH-meter and NH_3-electrode. For the re-set of pH, the samples were first cooled down to room temperature and subsequently re-set to a pH of 7.2 ± 0.2.

Statistical Analyses

To determine the significance of the results of the NH_3-N and pH measurement, the statistic software SPSS, version 20, from IBM was used.

The mean values of NH_3-N and the pH value were examined regarding significant differences between the different treatments and times of exposure. Start values of NH_3-N at time 0 were subtracted from the measured NH_3-N values after the treatment to focus on the increase of NH_3-N compared to the start. To be able to compare the amount of urea-N which was hydrolysed within the 21-23.5 hours after the exposure to the treatment, the NH_3-N values were measured at the end of the treatment (before being neutralized) and were subtracted from the final measured NH_3-N values. All the treatments were monitored over a 24 hours' period regardless of the treatment exposure, e.g. the re-activation times varied from 21-24 hours. To compare the reactivation rates within one time period, the values were extrapolated, assuming a linear hydrolysation of urea, to compare the amount of hydrolysed urea-N within a re-activation period of 24 hours for each treatment:

$$((NH_3\text{-N at 24 hours} - NH_3\text{-N after exposure time}) / (24 \text{ hours} - \text{exposure time})) * 24 \quad \text{(Equation 4)}$$

For each exposure interval and treatment, a MANOVA was performed, the significance was tested with Wilk's Lambda. To find all possible significantly different pairs of means, Tukey's honest significant difference (Tukey HSD) as post hoc test was included which creates subsets of treatments which are not significantly different (A, AB, B etc.). For determination of correlation, Pearson correlation was used. In case of suspicion that an error type II (a significance was not detected by the statistic program) occurred,

single ANOVAs were performed between two specific treatments to clarify the significance. In the overall comparison, further groups were created by time and treatment for example urine and faeces at pH 10 und a temperature of 60 °C at a time of 1 hour etc. An alpha value of 0.05 was chosen. There was no normal distribution in the raw data, neither in the logarithm and the residuals of the data but due to the controlled conditions of the experiments and the few repetitions, it was assumed that the data were normally distributed to be able to use MANOVA and ANOVA statistical tests. Further, the standard deviations (STDEV) differed between the treatments. MANOVA and ANOVA require both, a normal distribution and equal STDEV of the data. Thus, the statistical results given by MANOVA and ANOVA in this study were only used as a help in the identification process of differences between the treatments but the differences could be identified visually in the graphs.

RESULTS

The amount of hydrolysed urea-N, post-exposure (in 24 hours after treatment exposure) and during exposure, representing the urease activity and represented by the amount of NH_3-N, was compared between the treatments within experiments as well as in between all experiments. First, the results for each treatment of the single experiments are presented. Then, the treatments of all experiments were compared to each other. Since start values of NH_3-N were different for each urine, the increase of NH_3-N values compared to the start values were used to represent the amount of hydrolysed urea-N. The amount of hydrolysed urea is also given as fraction in percent of the amount hydrolysed in the respective control treatment UF. If not otherwise stated (e.g. with ANOVA or Pearson), the p values of the MANOVA were reported as a help for identifying significant differences between the results.

For the purpose of clarity, the labels for the treatments were used according to the following pattern:

U, UF	*Urine, Urine + Faeces*
T	*Temperature of the treatment (e.g. T60 = a treatment at 60 °C)*
pH	*pH of the treatment (e.g. pH10 = a treatment at a pH of 10)*

When either T or pH was not mentioned, the treatment did not include any manipulation of the respective factor. In case of a missing T, the treatments were conducted at room temperature (22 °C). When the pH was not manipulated, the urine used had an average start pH of 6.4 ± 0.4 and the pH increased over time due to the hydrolyses of urea.

Experiment 1: Heat treatment

The results of the first experiment, comparing the effect of temperatures of 60 (UF_T60), 80 (UF_T80), 95 °C (UF_T95) on urease activity in mg/L (and % compared to the control), represented by hydrolysed urea-N, are presented in the following.

Non-enzymatic urea hydrolysation by heat

The amount of NH_3-N increased in U_T80 and U__T95 by 51.4 (4.2 % compared to the control) and 190 mg/L (16 %) respectively after 3 hours compared to the start at time = 0 (270 mg/L, $p < 0.001$). U_T60 did not show a significant increase in initial NH_3 (+ 3.2 mg/L, 0.26 % on average compared to the control).

In the control samples without added faeces (U_T60, U_T80, U_T95), the pH stayed stable at a value of 6.5 ± 0.3 pH over the three hours of exposure at temperatures from 60, 80 and 95 °C.

Enzymatic urea hydrolysation at 60, 80 and 95 °C

Figure 6 illustrates the fraction of hydrolysed urea-N post-exposure of the three heat treatments compared to the respective control (plain filled) and the fraction of urea hydrolysed during the exposure, illustrated with a patterned fill.

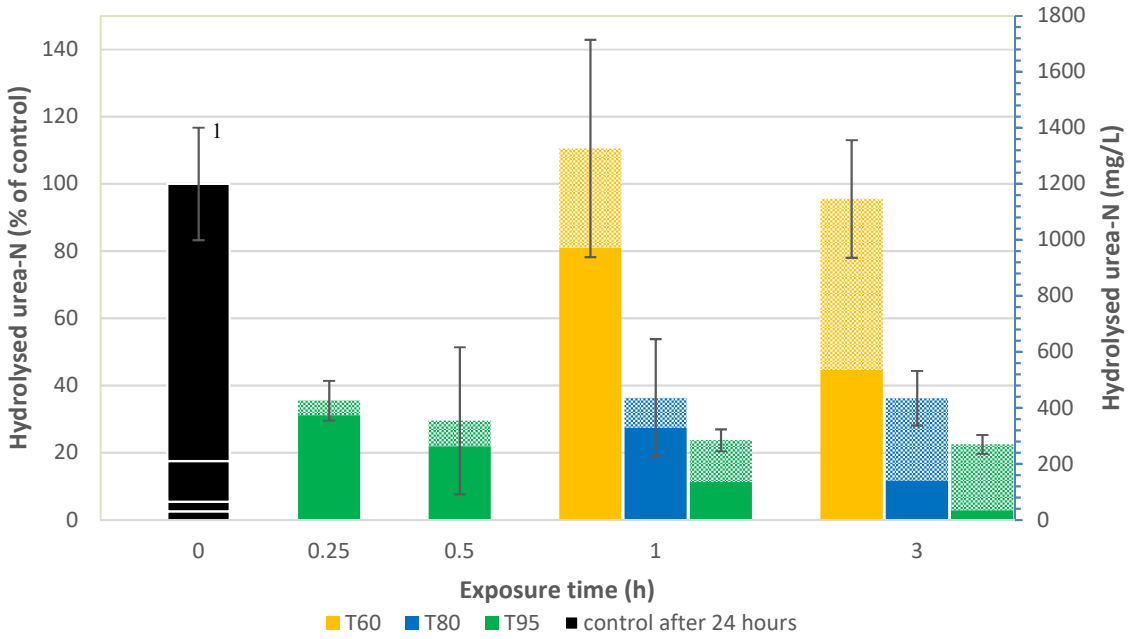

Figure 6. A comparison of the amount and fraction of hydrolysed urea-N between the treatments and exposure times, given in mg/L and in % compared to the respective control. White lines in the control bar (black) show the fraction of hydrolysed urea-N after 0.5, 1 and 3 hours. The solid filled bars show the fraction of hydrolysed urea-N in 24 hours post-exposure to 60, 80 and 95 °C. The pattern filled part of the bar illustrates the hydrolysed urea-N during the exposure to the heat. The STDEV is given for the total of hydrolysed urea. [1]The control for UF_T60 is not shown in the graph due to legibility. An average amount of 1400 mg/L urea-N was hydrolysed. Percentage of UF_T60 are given according to this control (UF (of T60)).

Due to reasons of time and schedule, the treatment UF_T60 was conducted on different days than UF_T80 and UF_T95, thus different faeces and urine samples were used which lead to a different amount of urea-N hydrolysation in the respective control (1400 vs 1200 mg/L). After an exposure to UF_T60 for 3 hours, the amount of urea-N hydrolysation post-exposure was lower (550 mg/L, 38 %) than in the respective control (UF (of T60), 1400 mg/L, $p < 0.01$). The treatment UF_T80 decreased the amount of urea hydrolysation post-exposure for 1 and 3 hours compared to the respective control UF (of T80 & T95) from 1200 to 330 mg/L (27 %) and 140 mg/L (12 %) urea-N respectively ($p < 0.001$).

In the treatment UF_T95, the amount of post-exposure urea-N hydrolysation decreased already after an exposure of 0.25 hours from 1200 to 380 mg/L (31 %, $p < 0.001$) but was still higher compared to after an exposure for 3 hours with 35 mg/L (2.9 %, $p < 0.05$) post-exposure. During the exposure for 3 hours at 95 °C, only slightly more urea-N was hydrolysed in UF_T95 than in the control treatment without faeces, U_T95 (240mg/L, 20 % vs 190 mg/L, 16 %).

Generally, after any exposure time to UF_T80 (140 - 330 mg/L, 12 – 27 %) and UF_T95 (35 - 380 mg/L, 2.9 – 31 %) the amount and fraction of urea-N hydrolysation post-exposure was lower than the post-exposure values after an exposure to UF_T60 (550 - 990 mg/L, 45 – 69 %) ($p < 0.001$).

Experiment 2: pH treatment

The results of the Experiment 2, comparing the effect of pH 10 (UF_pH10), 11 (UF_pH11), 12 (UF_pH12) and 13 (UF_pH13) on urease activity in mg/L (and % compared to the control), represented by hydrolysed urea, are reported in the following.

Non-enzymatic urea degradation by pH

The samples without added faeces, U_pH10, U_pH11, U_pH12 and U_pH13, did not change significantly in pH and NH_3-N within 24 hours compared to values at time = 0 (120 mg/L start + 0 - 30 mg/L during exposure, 0 – 2.8 % compared to the control UF).

Enzymatic urea hydrolysation at a pH of 10, 11, 12 and 13

The pH compared to the control (UF) increased in average from 6.4 to 8.8 within 24 hours. The pH of the treatment UF_pH10 and UF_pH11 decreased to an average of 9.4 and 9.7, while the pH of UF_pH12 and UF_pH13 decreased to an average of 11.4 and 12.5 respectively.

The pH dropped below 10 in the treatments UF_pH10 and UF_pH11, thus 460 (43 % compared to the control UF) and 320 mg/L (31 %) of urea-N was hydrolysed within 24 hours' exposure, while during an exposure for 24 hours to UF_pH12 and UF_pH13, 52 (5 %) and 6.4 mg/L (0.7 %) was hydrolysed.

Figure 7 illustrates the amount and fraction of urea-N hydrolysed during the exposure and post-exposure.

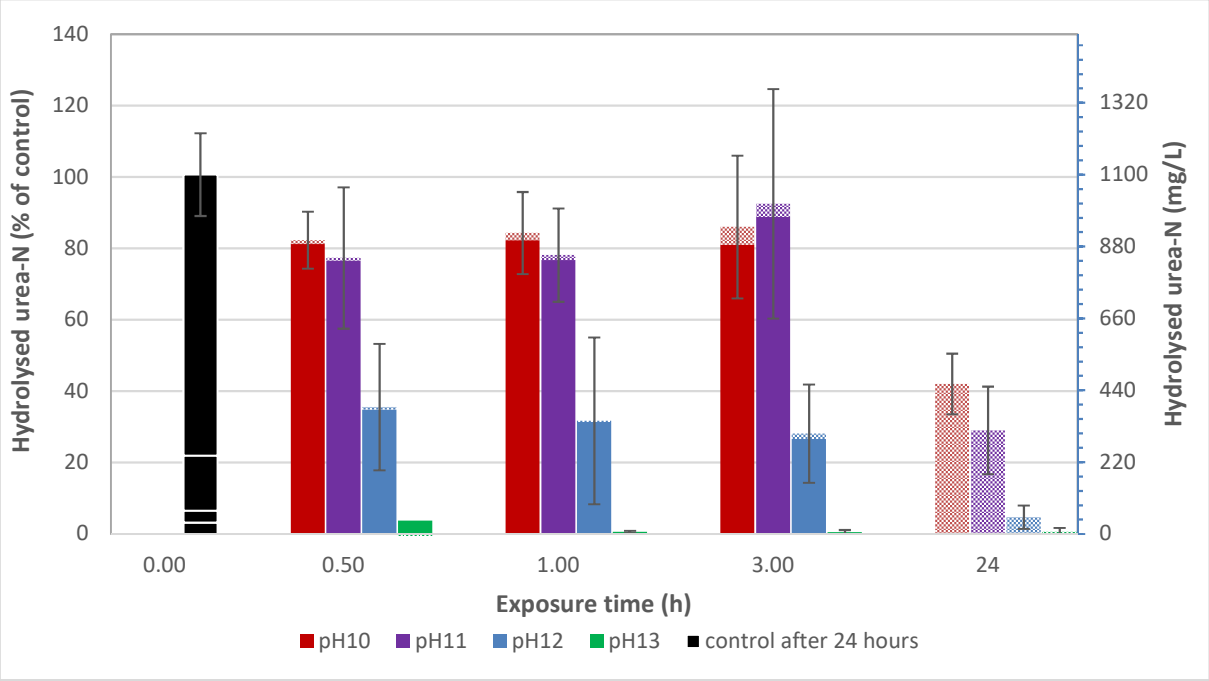

Figure 7. A comparison of the amount and fraction of hydrolysed urea-N between the treatments and exposure times, given in mg/L and in % compared to the control. White lines in the control bar (black) show the fraction of hydrolysed urea after 0.5, 1 and 3 hours. The solid filled bars show the fraction of hydrolysed urea-N in 24 hours post-exposure to a pH of 10, 11, 12 and 13. The pattern filled part of the bar illustrates the hydrolysed urea-N during the exposure time to the pH. The STDEV is given for the total amount of hydrolysed urea-N.

During exposure (illustrated by the patterned filled) every treatment significantly decreased the amount of hydrolysed urea-N compared to the respective time in the control treatment ($p < 0.05$ for pH 10 - $p < 0.001$ for pH 13), e.g. in 3 hours' exposure, the amount of hydrolysed urea-N decreased from 240 mg/L (22 %) in the control to between 0.7 (0.3 %, pH13) and 54 mg/L (23 %, pH10).

An exposure of the samples to UF_pH10 and UF_pH11 did not result in significant difference in amount of hydrolysed urea-N post-exposure (890 – 900 mg/L (~81 %) and 840 – 970 mg/L (76 – 89 %)).

Any time of exposure to UF_pH12 or UF_pH13 decreased the amount of hydrolysed urea-N post-exposure (ANOVA with control: $p < 0.001$ and $p < 0.05$): After an exposure to UF_pH12, the amount of hydrolysed urea-N decreased from 1100 mg/L in the control, to 380 (35 %), 340 (31 %) and 290 mg/L (27 %) for an exposure time of 0.5, 1 and 3 hours respectively. An exposure to UF_pH13 decreased the amount of hydrolysed urea-N post-exposure to 0 - 6.3 mg/L (0.58 %) which was lower than after UF_pH12.

Generally, after the exposure to UF_pH12 (290 – 380 mg/L, 27 – 35 %) and UF_pH13 (0 - 6.3, 0 – 0.58 %), the hydrolysation of urea-N post-exposure was lower than after the treatment UF_pH10 (890 – 900 mg/L, ~81 %) and UF_pH11 (840 – 970 mg/L, 76 – 89 %) (ANOVA: UF_pH12: both $p < 0.05$, UF_pH13: $p < 0.01$ and $p < 0.001$, compared to UF_pH10 and UF_pH11 respectively).

Experiment 3: Combined treatment

The results of the third experiment, comparing the combined effect of an exposure to 60 °C and a pH of 10 (UF_pH10_T60), 11 (UF_pH11_T60) and 12 (UF_pH12_T60) on urease activity in mg/L (and % compared to the control) are presented in the following.

Non-enzymatic urea degradation

The average pH value at start was 6.6 ± 0.4. The pH of samples U_pH10_T60, U_pH12_T60 and U_pH13_T60 decreased to 9.6, 10.2 and 11.4 within 24 hours respectively.

In those treatments, an average of 30 (1.42 % compared to the control UF), 35 (1.7 %) and 45 mg (2.1 %) of urea-N was hydrolysed in 24 hours of exposure after the start value of 160 mg/L NH_3-N ($p < 0.001$).

Enzymatic urea hydrolysation at a temperature of 60 °C and a pH of 10, 11 and 12

Similar to Experiment 2 the pH dropped with time from the start pH-values of 10, 11 and 12 (± 0.15). After 24 hours, a pH of 9.2, 9.7 and 10.4 in the combined treatments with 60 °C and pH 10, 11 and 12 was measured respectively.

All combined treatments significantly decreased the hydrolysation of urea-N post-exposure compared to the control (2100 mg/L) (Figure 8).

After 0.5 and 1 hour of treatment, a significant difference was found between the treatment UF_pH12_T60 (42 and 36 mg/L) to UF_pH10_T60 (570 and 150 mg/L, $p < 0.05$).

The exposure to the treatment UF_pH10_T60 for 3 hours decreased the amount of hydrolysed urea-N post-exposure by a greater amount (150 mg/L, 7.3 % compared to the control) than an exposure of only 0.5 hours (860 mg/L, 41 % $p < 0.05$). At the higher pH treatments (UF_pH11_T60 and UF_pH_12_T60), the difference was not significant between the times of exposure to the treatment (for UF_pH11_T60: 130 – 490 mg/L, 6.3 – 23 % and for UF_pH12_T60: 13 – 42 mg/L, 0.6 – 2 %). After an exposure time of 3 hours to UF_pH11_T60 and UF_pH12_T60, the difference between the treatments was not significant (130 and 13 mg/L, 6.3 and 0.6 %). The difference between UF_pH10_T60 (150 mg/L, 7.3 %) and UF_pH12_T60 (13 mg/L, 0.6 %) was still significant ($p < 0.05$).

During the exposure for 3 hours (patterned filled), the amount of hydrolysed urea-N was lower for both, UF_pH11_T60 (43 mg/L, 2 %, $p < 0.05$) and UF_pH12_T60 (5.3 mg/L, 0.25 %, $p < 0.05$) compared to UF_pH10_T60 (330 mg/L, 16 %).

The treatment UF_pH12_T60 of any exposure time decreased the amount of hydrolysed urea-N post-exposure, to the point that it was not significantly different to the control without added faeces, U_pH12_T60 ($p = 1$). Within 24 hours post-exposure, 45 mg/L (1.9 %) urea-N were hydrolysed in the control treatment and 13 (0.6 %), 36 (1.7 %) and 42 mg/L (2 %) urea-N were hydrolysed after an exposure of UF_pH12_T60 for 0.5, 1 and 3 hours respectively.

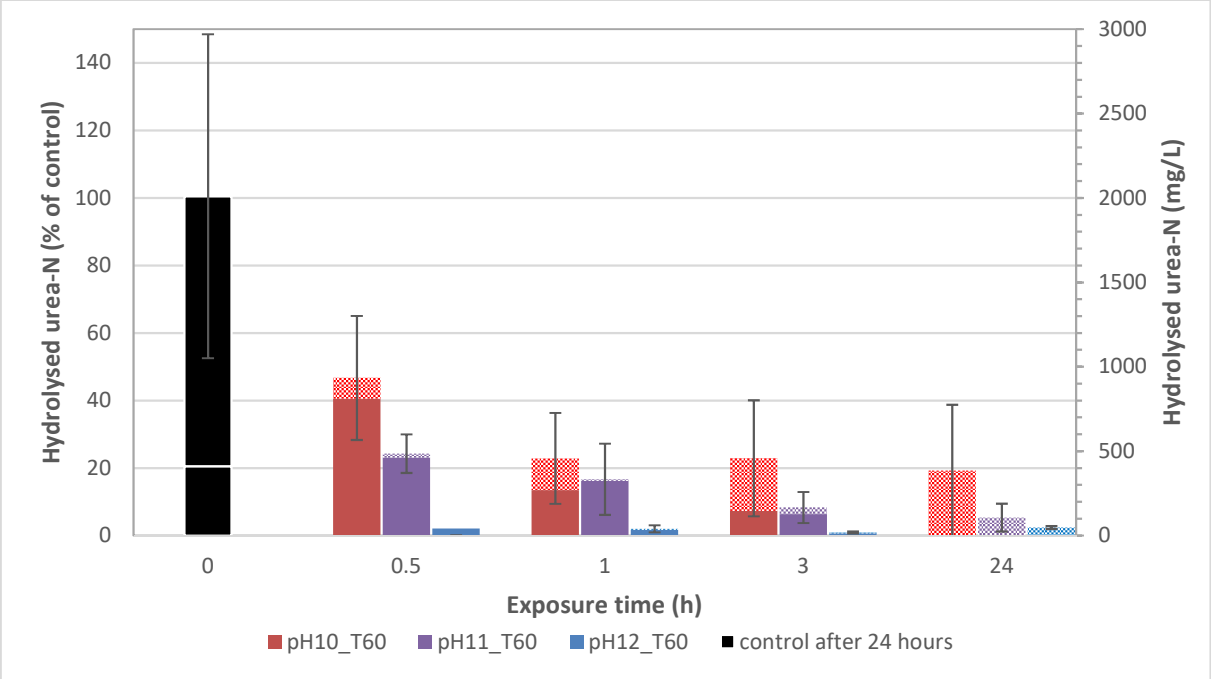

Figure 8. A comparison of the amount and fraction of hydrolysed urea-N between the treatments and exposure times, given in mg/L and in % compared to the control. The white line in the control bar (black) shows the fraction of hydrolysed urea after 3 hours. The solid filled bars show the fraction of hydrolysed urea-N in 24 hours post-exposure to a pH of 10 (pH10_T60), 11 (pH11_T60), 12 (pH12_T60) at 60 °C. The pattern filled part of the bar illustrates the hydrolysed urea-N during the exposure time to the pH. The STDEV is given for the total amount of hydrolysed urea-N.

A comparison of all treatments

To be able to compare all treatments, the comparison was done according to the percent (%) of NH_3-N compared to the respective control of the treatment.

Figure 9 illustrates the fraction of urea-N hydrolysed in the treatments for an exposure of 24 hours (% compared to the control). In all the here compared treatments, urine and faeces were added, but the label UF was skipped in the graph to allow a better legibility. The numbers for the heat treatments (T60, T80 and T95) were extrapolated from the 3-hour data (Equation 4, Statistical Analyses), a final pH of 9 was assumed according to the literature. Treatments including an alkaline pH showed significantly lower hydrolyses than the heat treatments (T60 – T80) of which T60 showed the highest hydrolyses. This was also shown at the shorter exposure times (0.5 – 3 hours, $p < 0.001$).

On average, 1700 ± 770 mg/L of urea-N was hydrolysed in the control (UF) for 24 hours. A comparison of the treatments reducing the urea-N hydrolysation to < 10 % compared to the control is illustrated in Figure 10. The smallest fraction of urea-N post-exposure was hydrolysed after any time of exposure to UF_pH13 (-2.9 – 0.58 %) or UF_pH12_T60 (0.6 – 2 %). These results were followed by UF_T95 for 3 hours (2.9 %) and UF_pH11_T60 or UF_pH10_T60 for 3 hours (6.3 and 7.3 %).

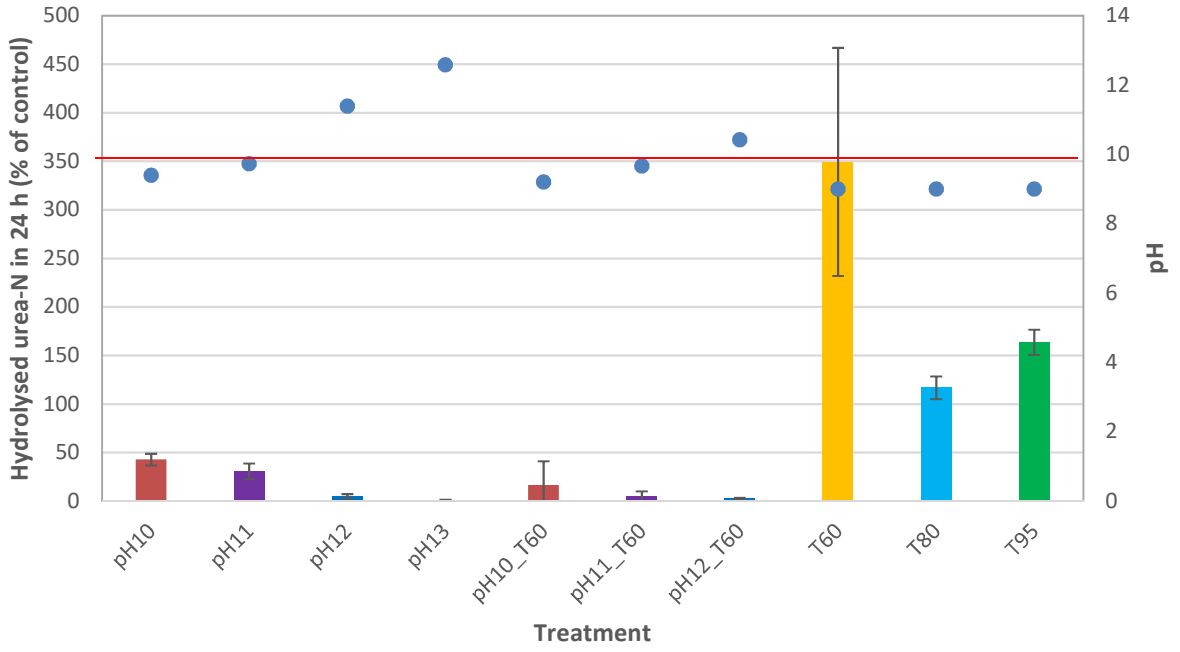

Figure 9. The fraction of hydrolysed urea-N in percentage of the respective control during exposure for 24 hours to the treatment and the pH (●). Data for heat treatment (T60, T80 and T95) are estimated from 3-hour data. The red line marks pH 10 below which urease is active if not irreversibly inactivated.

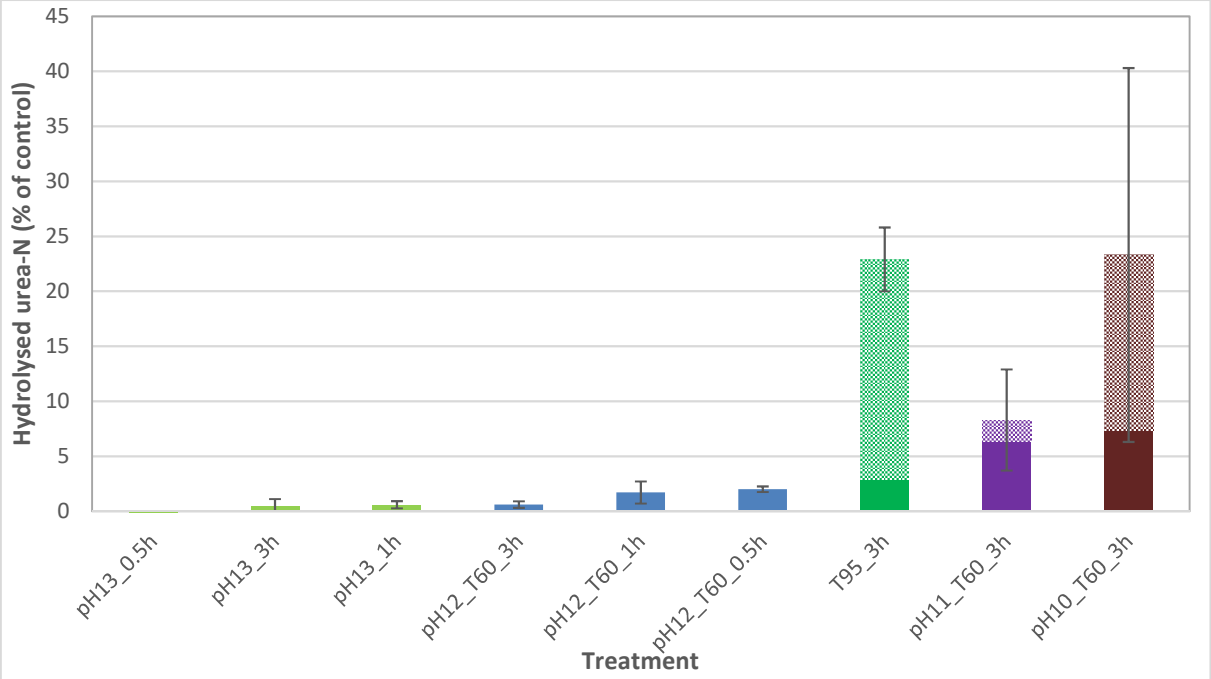

Figure 10. The treatments resulting in a urea-N hydrolysation post-exposure of < 10 % compared to the control. The fraction of hydrolysed urea-N during the exposure is illustrated with a patterned filled, the fraction of hydrolysed urea-N in 24 hours post-exposure is given in plain filled. STDEV is given for the total fraction of hydrolysed urea-N. When pH or temperature (T) is not given, it was not manipulated by the treatment. The last cipher indicates the exposure time.

Figure 11 a and b illustrate the urea-N hydrolysation between 11 – 30 % and 30 – 100 % respectively.

The largest fraction of hydrolysed urea-N post-exposure was determined in UF_pH10 (81 – 82%), UF_pH11 (76 – 89 %), regardless of the time of exposure, and UF_T60 for 1 hour (69 %) (Figure 11b). In more detail, the treatments are compared according to the exposure times in the following. STDEVs are given in Appendix I.

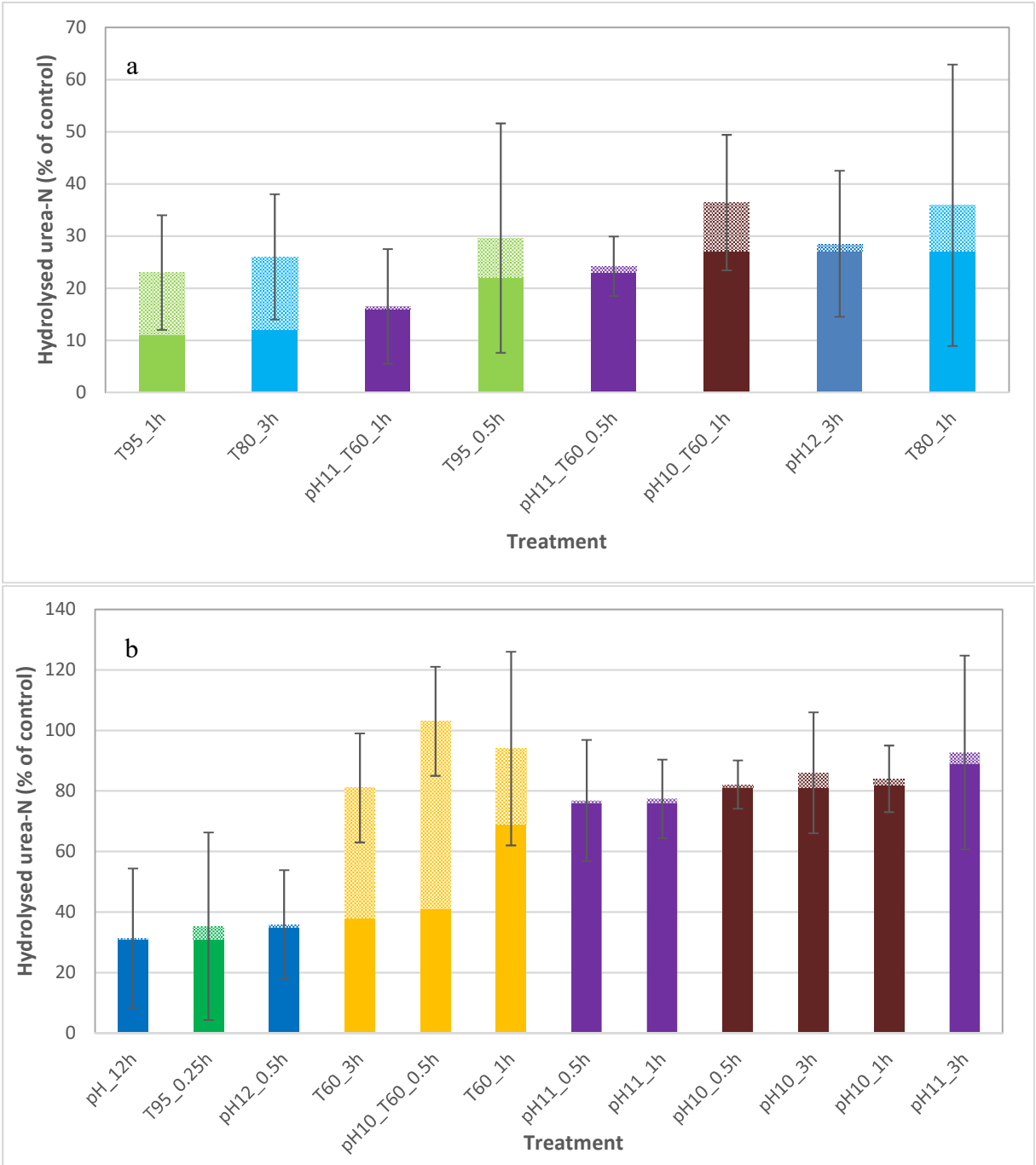

Figure 11a and b. The treatments resulting in a urea-N hydrolysation post-exposure between 10 - 30 % (a) and > 30 % (b) compared to the control. The fraction of hydrolysed urea-N during the exposure is illustrated with a patterned filled, the fraction of hydrolysed urea-N in 24 hours post-exposure is given in plain filled. STDEV is given for the total fraction of hydrolysed urea-N.

During the 0.5 hours of exposure to the treatment, the largest fraction of urea-N was hydrolysed during UF_T95 (7.6 %; Figure 11a) and UF_pH10_T60 (6.2 %; Figure 11b). Treatment UF_pH13 (-3.3 %; Figure 10) had the smallest fraction of hydrolysed urea-N. The heat treatments at 60 and 80 °C were not conducted at the exposure time 0.25 and 0.5 hours. In the 24 hours after treatment exposure for 0.5 hours, UF_pH13 showed the smallest fraction of hydrolysed urea-N (-2.9 %; Figure 10) whereas UF_pH10 and UF_pH11 showed the largest fraction (81 and 76 % respectively; Figure 11b).

During 1 hour of exposure, the largest fraction of urea-N was hydrolysed at UF_T60 (25 %; Figure 11b). In the 24 hours post-exposure for 1 hour, UF_pH13 and UF_pH12_T60 had the smallest fraction (0.58 and 0.6 %; Figure 10) whereas UF_pH10 and UF_pH11 had the largest fraction of hydrolysed urea-N (82 and 76 %; Figure 11b). During the 3 hours' exposure, UF_pH13 and UF_pH12_T60 had the lowest (0.07 and 0.25 %; Figure 10) while UF_T60 showed the largest hydrolyses (43 %; Figure 11b). In the 24 hours after exposure for 3 hours, the smallest fraction of hydrolysed urea-N was after an exposure to UF_pH13 (0.49 %; Figure 10), UF_pH12_T60 (0.6 %), UF_T95 (2.9 %), UF_pH11_T60 (6.3 %) and UF_pH10_T60 (7.3 %; Figure 10).

Follow-up tests

The amount of urea-N hydrolysation by urease in the collected faeces was compared to the amount of urea-N hydrolysation in the same amount (wet weight) of the biofilm sludge in a regular toilet bowl which was not flushed after urination but only after excretion of faeces (Figure 12). Results showed clearly that urea-N hydrolysation in 0.1 g of biofilm was significantly higher than in faeces ($p < 0.001$ after 1 hour, $p < 0.001$ after 3 hours and $p < 0.05$ after 24 hours).

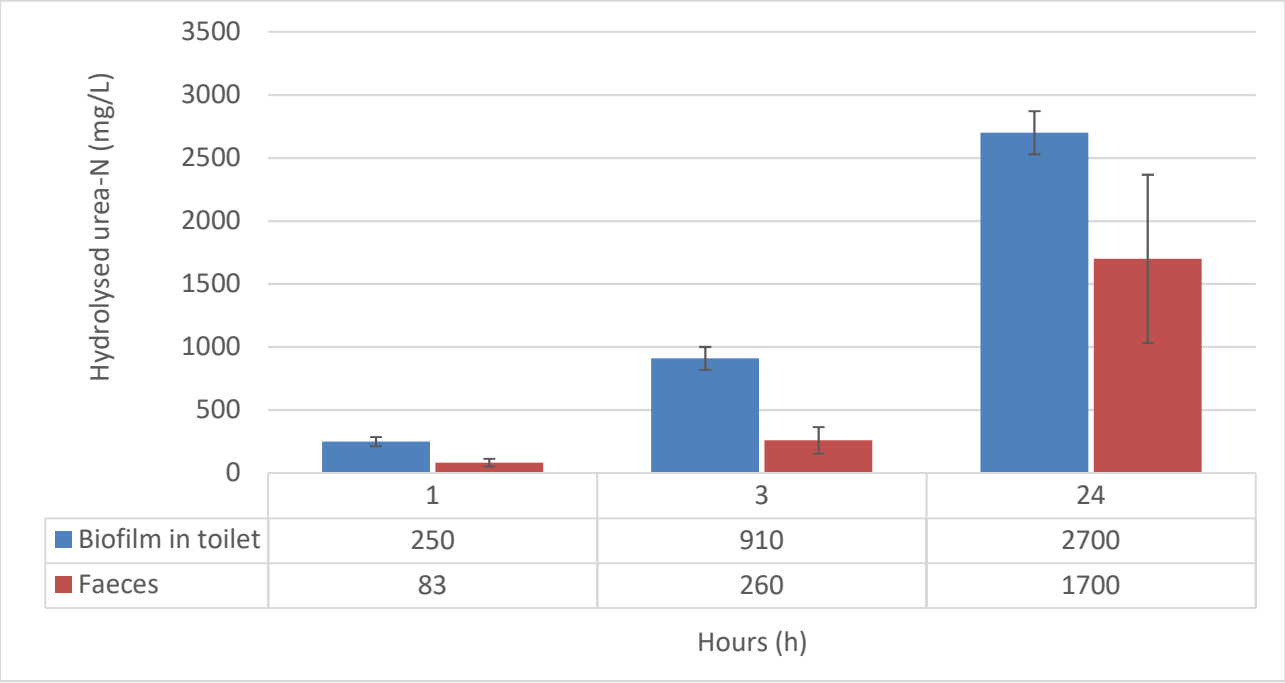

Figure 12. A comparison of the amount of urea-N hydrolysed over time by faeces and by the biofilm from a toilet bowl.

Further, it was tested if there was an overall correlation between the urea-N hydrolysation during and post-exposure of the treatment exposure and the start value of NH_3-N or the start pH. Taking into account all data presented in this study, a strong correlation was found between the start NH_3-N value ($p = 9 * 10^{-14}$, Pearson) and the start pH value as well as between the start NH_3-N values and the amount of urea-N hydrolysed during the exposure ($p < 0.005$, Pearson). No correlation in NH_3-N values could be stated

between the amount of urea-N hydrolysed post-exposure to the amount of urea-N hydrolysed during the treatment or the start NH_3-N values.

Considering this, in Experiment 1 (heat treatment), the urine samples had higher NH_3-N start values (270 mg/L on average) compared to the other treatments, whereas in Experiment 2 (pH treatment), urine had the lowest start value (120 mg/L on average, $p < 0.05$). Urine used in Experiment 3 (combined treatment) had medium start values of 160 mg/L which were not significantly different to the other treatments.

The range of the STDEV in total NH_3-N values of the treatments correlated with the amount of hydrolysed urea-N ($p < 0.05$, Pearson). The higher the value of hydrolysed urea-N, the higher was the STDEV.

DISCUSSION

The results verified that heat and pH have a strong influence on urea-N hydrolysation by urease enzymes. New treatment options were determined for an inactivation of urease enzymes to prevent loss of N in urine and simplify the concentration of nutrients. Some treatments detected in this study even led to an irreversible inactivation of urease which allows a much broader range of sanitation technologies with the aim to use human urine as valuable fertilizer.

Total NH_3-N values

To get the total amount of urea-N in this experiment, jack bean urease was applied to the collected urine. An average of 6 g of NH_3-N per L was measured, out of which 5.7 g was hydrolysed N, which is little less than reported by the literature (around 85 % urea-N: 6 – 7.7 g of 7 - 9 g/L N-tot (Kirchmann , 1994)). Those results are typical for the current lab group of Kretsloppsteknik, from which the urine was collected (Simha , 2016; Senecal , 2017). It might have been due to the preference of the collection group of a large amount of fluid intake during the day and that the urine collection did not include the morning urine which is the most concentrated, resulting in higher diluted urine (Jönsson , 2004, p. 7).

The effect of temperature on urease enzyme activity

The large fraction of hydrolysed urea-N at 60 °C (Figure 6) indicates a high urease activity from human faecal bacteria and support the findings about the optimal temperature for jack bean urease in literature (Eremev , 1999; Arica , 2000). Particularly the comparison of the pH treatment at 60 °C (UF_pH10_T60, Figure 8) and at room temperature (22 °C, UF_pH10; Figure 7) confirmed this pattern: during 3 hours at 60 °C and pH 10, urease was three times more active than at room temperature and a pH of 10 (5 % and 16 % compared to the control). In the control treatment U_pH10_T60 only 2 % of the urea-N was hydrolysed which signals that this increase was not due to non-enzymatical hydrolysation (Chapter: Results, Experiment 3: Combined treatment, Non-enzymatic urea degradation). The extrapolation of the 3-hour data to 24 hours (Figure 9) of the heat treatment of 60 °C resulted in 350 % urea-N hydrolysation compared to the control. This means that 5 g of urea-N corresponding to 88 % urea-N was hydrolysed of the total amount urea-N in the urine (5.7 g). Since a urease inactivation can be expected after some time, this extrapolation probably gave too high values. Nevertheless, it shows the severity of urea hydrolysation by urease at a temperature of 60 °C. The highest fraction of the large amount of hydrolysed urea in the 95 °C treatment is likely to be due to non-enzymatic degradation of urea-N. Extrapolated data from the control without faeces only showed slightly lower results (1900 vs 1500 mg/L; Chapter: Results, Experiment 1: Heat treatment, Non-enzymatic urea hydrolysation by heat) and urease was already inactivated to 31 % after a short exposure time of 0.5 hours in these experiments (Figure 6).

The lower STDEVs at temperatures 80 and 95 °C (Figure 6) confirmed that at temperatures above 80 °C, temperature is a limiting factor for urease activity, as reported previously (Krajewska , 2012). At this point,

the reaction of reversible dissociation/denaturation of urease towards irreversible association of denatured and native forms of the enzyme increases (Figure 13). However, the constant of the association reaction between denatured forms is by two orders of magnitude higher than the association reaction with the native form. The backward reaction from the reversible denatured form to the native form is still faster than the reaction from the native form to the denatured form, even at high temperatures (85 °C). This explains the high observed stability of urease (Illeová , 2003).

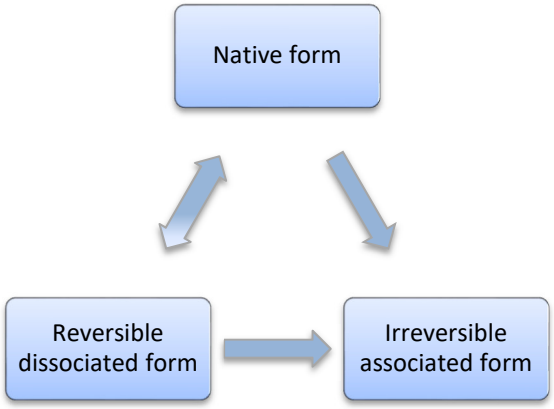

Figure 13. Urease enzyme inactivation. Urease dissociates reversibly into a denatured form which then can associate irreversibly with other denatured forms or native forms or turn back into its native form. The irreversible association between denatured forms occurs quicker than the association with native forms.

From the previous studies by Arica (2000), a faster inactivation could still have been expected at 60 °C than the one experienced in the presented study: Arica reported that only 5 % of free jack bean urease was still functioning after 120 min at 55 °C and all initial activity was lost after 60 min at 65 °C. In this study, after an exposure of 3 hours to 60 °C the amount of urea-N hydrolysed was only reduced to 45 % of the urea-N hydrolysation in the control treatment (UF; Figure 6). This is closer to the half-life of 120 min, reported by Krajewska et al. (2007) at a temperature of 70 °C. In their study, they still experienced 40 % of initial urease activity after 250 min. The high stability of urease also explains why the urea-N hydrolysation after a treatment of 80 °C for 1 hour was still 28 % compared to the control (Figure 6). After an exposure to 95 °C, the urea-N hydrolysation decreased quickly resulting in 31 % after 15 minutes' exposure. This was still high compared to a study by Baker & Mustakas (1972) which found no activity after cooking of soybean urease at 90 °C for 15 minutes. The lower inactivation rates in the experiment might have been caused by a certain heating up time until the samples reached the respective temperature in the heat block. Although the water filled heat block should have transferred the heat quickly, measurements showed that it took around 7 minutes to heat up a sample to 80 °C in the 95 °C treatment. A heating up time will also need to be taken into account in real treatment settings.

The effect of pH on urease enzyme activity

A higher pH leads to a change of the ion state in enzymes. If the active site of the enzyme is in the wrong ionized state, enzyme functions decrease (Frankenberger and Johanson , 1982). Although, much literature exists about the inactivation of urease at high pH, no literature was found about the reversibility of this inactivation. This study indicated a new hypothesis that at a pH of 13 an irreversible inactivation occurs and that a significant decrease of enzyme activity after exposure to a pH of 12 can be expected (Figure 7). The urea-N hydrolysation in the samples exposed to pH 12 and 13 decreased to 27-35 % and ~0.5 % compared to the control respectively. In these experiments, it was shown that the inactivation of urease enzyme by alkaline pH was happening within 0.5 hours. The exact kinetics of this inactivation should be modelled in further studies.

Regarding the upper limit of urease activity at alkaline pH, this study verified that there was no urease activity above an alkaline pH of 10 (Figure 7). Randall et al. (2016) reported an upper limit of urease activity of pH 11 whereas Kabdaşlı et al. (2006) set the upper limit at pH 10. In this study, urea-N hydrolysation was observed during the treatment UF_pH11 for 24 hours (Figure 9). This can be explained by the decrease of the pH in this treatment to a pH of 9.7 within the 24 hours due to addition of faeces, natural processes such as degradation of the organic matter and the high buffer capacity of urine at a pH of 9. The drop of the pH below 10 might have allowed the reactivation of the urease in this treatment. In an open system, interaction with CO_2 in the air could speed up the drop of the pH.

Urease activity in the bigger context

The comparison of urea-N hydrolysation from faecal sources and from the biofilm build up in toilet bowls showed that urease activity was higher when derived from the toilet bowl (Figure 12). These results might be explained by a larger concentration of bacteria with high urease activity in the biofilm. Whereas faeces contains a low dry mass of only 22 % (Vinnerås , 2002) and 10^{10} microorganisms per gram dry matter, (Lentner and Wink , 1981); the build-up (including precipitates and biofilm) consists of 61 % dry mass, mostly precipitates such as struvite, apatite and calcite (Udert et al. 2003), where bacteria, specialised on high urease activity are attached to (Oki , 2010). The high urease activity in this build-up stresses the need to inactivate urease directly after excretion, and before any pipe transport, otherwise urea-N can be hydrolysed and N potentially lost by evaporation and thus not be available for fertiliser use.

There is a large variation of urease enzymes in the gut flora which was represented in the high STDEV of 40 % (± 770 mg/L) in the amount of hydrolysed urea-N after 24 hours in the control treatment. This large variation was caused by an influence of numerous endogenous and exogenous factors including diet as well as infections, allergies, xenobiotics and other drugs (Lynch , 2016). For example, David et al. (2013) determined the intestinal microbiome of two groups following an either animal-based or plant-based diet to significantly differ already after 5 days. The activity of faecal urease reversibly decreases by up to 66 % when consuming an uncooked vegan diet (Ling and Hänninem , 1991). The variation in diet was represented in the diet of the presented collection group. There were three persons following a vegetarian diet, several persons whose diet contained little meat and some which had a meat-based diet. As the collection of faeces and urine was conducted anonymously and the collected faeces could not be tracked back to the person, it was not possible to incorporate this variation in this study. The variation in diets (and urease activity) was attempted to be nullified by using random faeces for each of the trials. More samples would have helped to decrease this variation, but faecal donations were difficult to collect daily since fresh faeces needed to be brought to or collected at university to obtain still fully active urease from the faeces. Between 8 to 12 different people were donating faeces regularly and the average number of fresh faecal samples collected daily was three. Hence there was still large variation in the urease activity rate.

Limitations of this study

In the results, p-values are given in many places. These should however only be taken as indications, as the requirements for strict statistical test on normal distribution and similar variance between treatments were not met.

It was tried to limit the variations in urine and faeces samples as much as possible by subtracting the start NH_3-value from the results and comparing the urea-N hydrolysation after the treatments in % of the respective control (Chapter: Statistical Analyses). That no correlation was found between the start NH_3-N values and the NH_3-N values of the 24 hours post-exposure confirmed that the different urine samples did not affect the hydrolysation of urea-N post-exposure. However, the correlation between start-values and amount of NH_3-N during the exposure indicated that the composition in the urine might have affected the urea-N hydrolysation during exposure. The STDEV of the start NH_3-N value in the collected urine was large

(48 % = ± 87 mg/L) which was due to several factors like diet and sport affecting the fractions of the composition of nitrogen compounds and urea released in the urine (Calloway and Margen , 1971; Dohm *et al.* , 1982; Bos *et al.* , 2003). According to Mackenzie (1986), NH_3 and urea are related in the kind that NH_3 directly stimulates ureagenesis in the human body to prevent hyperammonemia in the blood. A different composition of N compounds might explain the correlation between the start NH_3-N value and the amount of NH_3-N measured during the treatment exposure: different structures of compounds show different stabilities regarding heat and pH and release different amounts of NH_3-N during degradation (Sohn and Ho , 1995; Bos , 2003).

The amount of hydrolysed urea-N was measured after a total time of 24 hours consisting of treatment exposure and time post-exposure. To be able to compare the treatments regarding the amount of hydrolysed urea-N post-exposure, measured results were extrapolated to a common time of 24 hours after exposure.

The extrapolation of the post-exposure data (Equation 4) from 21-23.5 to 24 hours is not expected to falsify the results as enough urea was left to allow further urea hydrolysation by urease enzyme and the time of extrapolation was very little (between 0.25-3 out of 24 hours). Hotta & Funamizu (2008) detected a strong inhibition of NH_3 on faecal urease activity at pH > 8 and an amount of > 100 mg/L free nitrogen which was reached after 3 days in their study. However, hydrolysed and undiluted urine is proven to inactivate bacteria at a fast rate of hours to a few days (Vinnerås *et al.* , 2008) and urease has a half-life of around 110 hours at neutral pH and a temperature of 25 °C (Qin , 1994). Inactivation of the bacteria by exposure to urine and following degradation over time of the urease enzyme might explain the decrease in urease activity in the study by Hotta & Funamizu, (2008) rather than a strong product inhibition by NH_3. However, this needs to be confirmed in future research. More faeces were added in this study (20 g/L compared to maximum of 12.5 g/L in their study) which decreases the product inhibition effect of the ratio mol NH_3 to g urease. Qin & Cabral (1994) reported that no serious inhibition by free NH_3 can be expected up to 0.04 M (560 mg/L). In the presented experiments, the start NH_3-N value was 200 mg/L on average (0.015 M). In the control after 24 hours, NH_3-N values as high as 4300 mg/L (0.31 M) were reached whereas the highest extrapolated value was 1600 mg/L (0.11 M). Further studies with more frequent measurements need to clarify if there might have been a significant effect of product inhibition for faecal urease in the presented study.

Even if the realistic values of the post-exposure data in this experiment would have been higher than the calculated results which reached an NH_3-N value of > 560 mg/L (limit of amount of NH_3 and thus significant product inhibition of urease enzyme by NH_3, reported by Qin & Cabral 1994), it did not affect the overall trend of the results. This study focused on optimal options for urease inhibition and the suggested treatments (Figure 9 and following chapter: Future perspectives of this study) did not exceed that threshold for product inhibition. Alternative tables giving the actual measured NH_3-N values, are attached in the appendix (The raw data for Experiment 1: Heat treatment.). In the open system of a urine-separating wastewater treatment, the favourable condition of product inhibition of urease enzymes by NH_3 will be reduced since produced NH_3 will become volatile and leave the system (equilibrium between dissolved NH_3 and gaseous NH_3 (Snoeyink , 1980)).

Future perspectives of this study

The most promising treatments with less than 10 % urea-hydrolysation, as shown in Figure 9, were the pH 13 treatments and the combined treatment with pH 12 at 60 °C. Although, the heat treatment at 95 °C for an exposure for 3 hours showed good results in the irreversible inactivation of urease activity post-exposure, the loss of urea-N during the exposure was large and needs to be taken into account when considering the treatment options. Further, a large energy consumption might be needed for an exposure at a temperature of 95 °C for 3 hours compared to a temperature of 60 °C which is often considered as a “waste-temperature” from heating of house or water (personal communication with Björn Vinnerås). Since ion-exchange of urine requires a high ion-exchange capacity of the respective ion-exchange medium and a frequent regeneration, a

combined-treatment of a pH of 10 or 11 with a heat-treatment of 60 °C for 3 hours should be considered as well.

The results of this study suggested to anion-exchange the urine to a pH of 13, using a strong-base resin directly at the toilet bowl and before it is piped, stored or further treated. A pH of 13 irreversibly inactivated all urease activity (Figure 7) and would allow a pipe transport to a central WWTP where the urine can be stored and dried centralized without greater N losses to produce a highly valuable fertilizer (Figure 14). Nevertheless, it needs to be assured that there is no second contamination during the pipe flow or storage if the pH drops below 10. According to Simha (2016), 100 % of the urine must interact and pass through a resin of 1.2 eq./L exchange capacity to achieve a pH of ≥ 12.5 with a 20 % volume to volume ratio of resin to urine. The loss of NH_3-N due to natural urea degradation during the high pH is expected to be low as in this study no significant non-enzymatical hydrolysation of urea-N was found in the controls without faeces addition in the pH treatments (Chapter: Experiment 2: pH treatment). Using resin would be attractive because it is low energy but the resin needs to be replaced or regenerated frequently to maintain its anion-exchange capacity (Landry *et al.* , 2015). A resin with higher exchange capacity or better regeneration efficiency might be more expensive but can result in lower treatment costs (Tarpeh *et al.* , 2017). Further, struvite precipitation in the pipe and thus clogging of the pipe might cause problems as struvite precipitation was also observed in the test tubes at a pH ≥ 11 in this study. If the pH can be maintained above a pH of 10 during the urine processing, irreversible urease inactivation is not needed. Thus, as long urine drying is combined with a pH above 10 and the stability of the pH is secured, it is feasible without higher losses of urea. This could be achieved using a drying medium with a pH > 10.

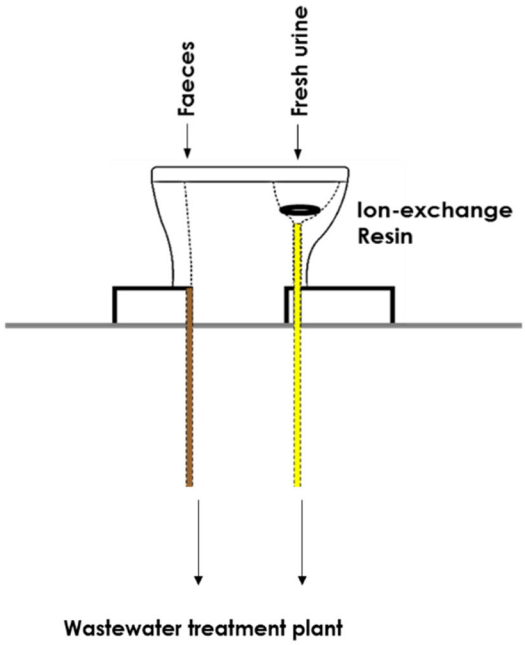

Figure 14. The collection of faeces and urine in a urine-separating toilet. The ion-exchange resin could be placed directly on the bottom of the toilet bowl, preventing any urease activity during the pipe transport (modified after Simha (2016)).

The suggested alternative to the ion-exchange resin would be the implementation of a combined treatment during the drying procedure, using a drying medium with a pH ≥ 10 and a drying temperature ≥ 60 °C. However, temperatures should not be increased to ≥ 80 °C since high losses of urea can be expected due to non-enzymatical degradation and evaporation of NH_3 as shown in Figure 9 and discussed in Chapter: The effect of temperature on urease enzyme activity). Several studies have been conducted about suitable drying media such as biochar, ash and lime (Dutta and Vinnerås , 2016; Simha , 2016). Biochar, for example, has the advantage of highly porous media, retaining important nutritive cations and anions, reduces leaching in soils and can increase plant yield particularly in tropical soils (Atkinson *et al.* , 2010). Those properties can

be advantageous for the urine-drying rate, retaining N, and, depending on the biochar source, it can reach an alkaline pH of 9.9 (Chan *et al.* , 2008). Additives such as KOH need to be used to increase the pH further (>10), at the same time serving as additional nutrients for plants (Simha , 2016). Different drying media and additives vary in their fertilizer compounds, thus could be chosen according to the soil and the crop which is planned to be fertilized with it. Lime is a frequently used media to mediate the acidity in soils. Wood ash with a pH of 11 combined with stored urine was successfully used as tomato fertilizer (Pradhan *et al.* , 2009). The availability of the nutrients needs to be evaluated in the drying-media-urine mix to make sure the fertiliser product provides the plant with available nutrients. Further, it is necessary to examine the polluting potential of the drying media when applied onto the field, as lime and wood ash from wood, which was painted or varnished, can contain heavy metals (Demeyer et al. 2001; Nicholson et al. 2003). As the urine is not ion-exchanged in this alternative, this approach would require a local urine drying system since during pipe transport high losses by the formation of the highly active biofilm in the pipes are expected (Oki , 2010) (Figure 15). A local urine-drying unit in the toilet would also allow an easy implementation into the houses without the need of installing an extra urine-pipe. Housing companies consider this as a big advantage (personal communication with Björn Vinnerås).

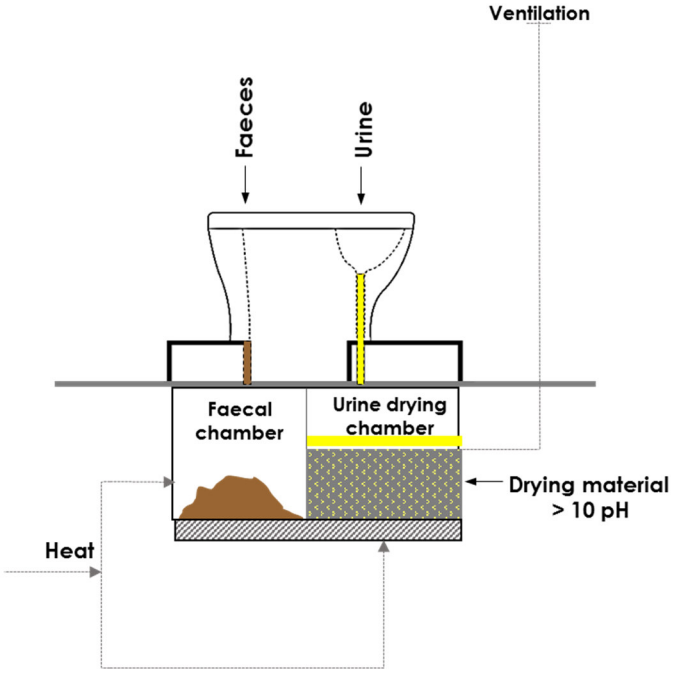

Figure 15. The collection of faeces and urine in a urine-separating toilet with a local urine-drying system (Simha 2016). The high pH > 10 in the drying medium could prevent any urease activity. An optional heating > 60 °C to increase the drying rate could eventually also lead to the irreversible inactivation of urease activity.

Losses can occur during the treatment until the complete inactivation of urease if the pH drops below 10. An eventual drop in pH is likely due to the influent urine with a pH of 6.4 on average (own measurements), the urea hydrolyses, the formation of NH_3 (weak base) and the thermal degradation of organic compounds in urine resulting in the formation of carbonic acids (Putnam , 1971; Simha , 2016). Simha (2016) found that in his urine drying system, the pH of 125 g biochar, with addition of KOH pellets in a weight ratio of 1:4, obtaining a starting pH between 12.5 and 13, decreased to a pH of 10.5 within 7.5 days with a urine loading of 50 ml of anion-exchanged urine (around pH 11.5) per treatment. Trade-off options would need to be evaluated between a frequent change of drying medium in the toilet or including an ion-exchange resin to ≥ 12 in the toilet bowl. The increase in pH to > 12 of the urine in the toilet bowl might mitigate a drop of pH in the drying media and inactivate urease activity during the drying process. Further studies are needed to

prove this hypothesis and compare the results with results of drop in pH in the drying media by non-ion-exchanged urine.

When the treatment options which include a manipulation of the pH, are applied to field studies, the pH would likely not be set back to a neutral pH but would decrease slowly over time. It needs to be examined how fast the pH decreases in storage tanks or during pipe flow to prevent a drop of the pH below 10. Studies by Simha (2016) showed that the pH of ion-exchanged stored urine decreased from 11.5 to below a pH of 10 within 7 days at 50 °C. According to the time and length of required pipe flow and storage, the pH could then be chosen to be lower than 12 or 13 without the risk of urease activity if a second contamination with urease enzymes can be prevented. This would reduce the frequency of regenerating the ion-exchanger. If a longer pipe transport or storage is required, urease would need to be inactivated irreversibly beforehand using pH ≥ 12 and a second contamination must be prevented.

CONCLUSIONS

This study has shown potential approaches to retain nitrogen in human urine with the aim to concentrate the nutrients in the urine to use it as a valuable fertilizer for plants. The presented results of a three times higher urease activity at 60 °C emphasize the need to inactivate the urease enzyme in urine-drying systems but showed that above a pH of 10, no urea-hydrolysation by urease occurred. Although, urease has shown an ability to maintain 30 % of its activity after exposure to a high temperature of 80 °C for 1 hour and regain around 30 % of its activity after being exposed to a pH of 12, efficient treatments for urease inactivation were found. Particularly the inexistence of any urease activity after an exposure to a pH of 13 and the considerable success of urease inactivation at the combined pH and heat treatments could give a direction towards new system options: Although urease regained its full activity after the exposition of urease to a pH of 10 at room temperature, an exposure to a combination of pH 10 with 60 °C for 3 hours decreased urea hydrolysation to 6.3 % post-exposure while at the same time preventing urease activity during the exposure due to the alkaline pH. It was shown that a combination of pH 12 and 60 °C completely inactivated urea hydrolysation by urease. Thus, for an irreversible inactivation of urease, the results of this study suggest a treatment of ion-exchange urine to a pH of 13 for a pipe-transport or a combined treatment with pH 12 and 60 °C for local urine drying systems. Due to the inactivity of urease enzymes above a pH of 10, an irreversible inactivation might not be necessary in every case, lower pH values can be considered as long as a drop of pH below 10 is prevented. It is expected that the presented results will significantly affect the development of future approaches to urease inactivation and help to develop suitable urine drying techniques, even though the p-values presented should only be taken as indications, as the requirements for strict statistical tests on normal distribution and similar variance between treatments were not met.

Future Research

Before implementing the treatments, more research is required and several factors need to be considered. The suggested treatments need to be observed in field studies with natural processes in urine-separating toilets, particularly including factors like pipe transport, ventilation and actual drying in an open system. It needs to be examined how fast the pH decreases in storage tanks or during pipe flow to prevent a reactivation of urease. A larger study for the best treatment options with more frequent measurements could help to identify the half-life of urease and the time when all urease activity is inactivated irreversibly. Suitable ion-exchange and urine-drying media need to be identified and the required reactor volume for the drying medium should be evaluated. The application of those urine-drying media regarding its fertilizer potential after treatment have to be evaluated. Finally, life cycle assessments could compare the proposed options with conventional fertiliser production, including the reduced load for WWTPs, to determine its environmental benefits and the potential competitiveness.

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APPENDIX

Appendix I. Comparison of all treatments.

Urine + Faeces	Time	Urea-N hydrolysed during treatment exposure (mg/L)	Urea-N hydrolysed during 24 hours after treatment (mg/L)	% of control	STDEV (±)	STDEV (%)
pH13	0.5 h	-36 (-3.3 %)	-26	-2.9	5.8	0.17
pH13	3 h	0.73 (0.07 %)	5.3	0.49	9.2	0.62
pH13	1 h	-1.6 (-0.1 %)	6.3	0.58	4.3	0.33
pH12_T60	3 h	5.3 (0.25 %)	13	0.6	17	0.3
pH12_T60	1 h	6.4 (0.3 %)	36	1.7	19	1
pH12_T60	0.5 h	-4.5 (-0.2 %)	42	2	17	0.25
T95	3 h	240 (20 %)	35	2.9	35	2.9
pH11_T60	3 h	43 (2 %)	130	6.3	70	4.6
pH10_T60	3 h	330 (16 %)	150	7.3	200	17
T95	1 h	150 (12 %)	140	11	40	11
T80	3 h	180 (14 %)	140	12	99	12
pH11_T60	1 h	10 (0.5 %)	340	16	210	11
T95	0.5 h	93 (7.6 %)	270	22	17	22
pH11_T60	0.5 h	26 (1.2 %)	490	23	110	5.7
pH10_T60	1 h	200 (9.4 %)	570	27	180	13
pH12	3 h	17 (1.5 %)	290	27	170	14
T80	1 h	110 (8.9 %)	330	27	99	27
pH12	1 h	4.2 (0.38 %)	340	31	250	23
T95	0.25 h	53 (4.3 %)	380	31	73	31
pH12	0.5 h	9.2 (0.84 %)	380	35	190	18
T60	3 h	620 (43 %)	550	38	210	18
pH10_T60	0.5 h	130 (6.2 %)	860	41	300	18
T60	1 h	360 (25 %)	990	69	390	32
pH11	0.5 h	9.2 (0.84 %)	840	76	220	20
pH11	1 h	16 (1.4 %)	840	76	150	13
pH10	0.5 h	11.8 (1.1 %)	890	81	97	8
pH10	3 h	54 (5 %)	890	81	270	20
pH10	1 h	22 (2 %)	900	82	140	11
pH11	3 h	40 (3.7 %)	970	89	400	32

When pH or temperature is not given, it was not manipulated within the treatment.

Appendix II. The results of 24 hour' treatment exposure. Data for heat treatments are estimated.

Group	mean pH	mean $NH_3$2	mean %	STDEV (mg/L)	STDEV (%)
U_pH10	9.8	5.7	0.5	7.4	0.7
U_pH11	11.0	6.0	0.5	5.2	0.5
U_pH13	13.1	5.9	0.6	1.5	0.2
UF_pH13	12.6	6.4	0.7	12	1.2
U_pH10_T60	9.6	30	1.3	27	1.0
U_pH11_T60	10.2	35	1.5	35	1.1
U_T60		25	1.8	44	3.1
U_pH12_T60	11.4	45	1.9	40	1.1
UF_pH12_T60	10.4	50	2.5	16	0.7
U_pH12	12.0	48	4.1	61	5.2
UF_pH11_T60	9.7	110	4.9	87	1.9
UF_pH12	11.4	52	5.0	36	4.1
UF_pH10_T60	9.2	410	16	410	9.7
U_T80		410	29	57	4.1
UF_pH11	9.7	320	31	130	16
UF_pH10	9.4	460	43	100	14
UF	8.8	1700	100	670	40
U_T95		1600	110	220	16
UF_T80		1400	120	200	12
UF_T95		1900	160	220	52
UF_T60		5000	350	200	100

Appendix III. The raw data for Experiment 1: Heat treatment.

Treatment	Deactivation time (h)	pH	Amount of hydrolysed urea-N (mg/L)	STDEV (mg/L)	pH after 24 hours	Amount of hydrolysed urea-N (mg/L) after 24 hours	STEDV (mg/L)
U_T60	1.00	6.4	0.0	0.0			
U_T60	3.00	6.4	3.2	4.5			
U_T81	1.00	6.3	10.5	10.4			
U_T82	3.00	6.5	51.4	7.1			
U_T95	0.25	6.3	10.1	18.0			
U_T95	0.50	6.4	37.8	22.9			
U_T95	1.00	6.5	77.2	28.7			
U_T95	3.00	6.8	190.8	40.6			
UF (of T80&T95)	0.25	6.3	23.4	9.9			
UF (of T80&T95)	0.50	6.3	30.6	3.7			
UF (of T80&T95)	1.00	6.4	64.8	19.6			
UF (of T80&T95)	3.00	6.8	212.9	84.2			
UF (of T80&T95)	24.00	6.1	1221.7	201.3			
UF (of T60)	1	6.7	111.9	28.3			
UF (of T60)	3	6.8	220.5	24.8			
UF (of T60)	24	8.6	1432.1	314			
UF_T60	1.00	7.5	361.6	50.9	8.7	1309.3	82.67
UF_T60	3.00	8.1	621.9	200.0	8.5	1099.0	31.01
UF_T80	1.00	6.6	108.4	29.1	7.4	421.2	12.60
UF_T80	3.00	6.8	176.1	24.5	7.0	301.5	35.11
UF_T96	0.25	6.4	52.8	24.4	7.5	429.9	67.47
UF_T97	0.50	6.5	93.2	19.8	7.2	355.0	80.90
UF_T98	1.00	6.6	151.7	37.2	7.0	283.8	262.51
UF_T99	3.00	6.8	239.5	22.2	6.9	270.5	250.74

Appendix IV. The raw data for Experiment 2: pH treatment.

Treatment	Deactivation time (h)	pH	Amount of hydrolysed urea-N (mg/L)	STDEV (mg/L)	pH after 24 hours	Amount of hydrolysed urea-N (mg/L) after 24 hours	STDEV (mg/L)
U_pH10	3.00	9.9	-15.8	29.6			
U_pH10	24.00	9.8	-0.9	9.8			
U_pH11	3.00	10.9	-9.5	6.5			
U_pH11	24.00	10.7	4.1	4.9			
U_pH12	3.00	12.0	-4.9	0.0			
U_pH12	24.00	12.0	30.6	53.1			
U_pH13	3.00	13.0	-49.8	63.4			
U_pH13	24.00	13.1	5.9	1.5			
UF	0.50	6.7	34.7	5.6			
UF	1.00	6.8	71.2	8.6			
UF	3.00	7.6	240.6	46.1			
UF	24.00	8.8	1095.0	125.7			
UF_pH10	0.50	9.7	11.8	10.3	8.8	882.4	87.7
UF_pH10	1.00	9.8	21.7	12.5	8.8	885.1	125.9
UF_pH10	3.00	9.7	54.2	39.6	8.8	830.0	219.2
UF_pH10	24.00	9.4	461.2	93.2			
UF_pH11	0.50	10.4	9.2	8.4	8.9	829.1	216.7
UF_pH11	1.00	10.3	15.6	17.9	8.8	820.6	143.0
UF_pH11	3.00	10.2	40.4	38.1	8.8	890.8	352.1
UF_pH11	24.00	9.7	322.4	134.4			
UF_pH12	0.50	11.8	9.2	8.5	8.2	381.7	193.9
UF_pH12	1.00	11.6	4.2	15.9	8.2	332.9	255.7
UF_pH12	3.00	11.6	16.7	10.5	8.1	271.6	150.6
UF_pH12	24.00	11.4	51.9	35.9			
UF_pH13	0.50	13.0	-35.8	53.4	7.3	3.4	1.9
UF_pH13	1.00	13.0	-1.5	5.9	7.9	4.5	3.6
UF_pH13	3.00	13.0	0.7	5.3	7.2	5.4	6.8
UF_pH13	24.00	12.5	6.4	12.0			

Appendix V. Raw data of Experiment 3: Combined treatment.

Treatment	Deactivation time (h)	pH	Amount of hydrolysed urea-N (mg/L)	STDEV (mg/L)	pH after 24 hours	Amount of hydrolysed urea-N (mg/L) after 24 hours	STDEV (mg/L)
U_pH10	24	9.6	30.1	26.6			
U_pH11	24	10.2	35.4	34.8			
U_pH12	24	11.4	44.8	40.1			
UF	0	6.7					
UF	3	8.5	407.1	31.6			
UF	24	9.0	2112.9	962.3			
UF_pH10	0.5	9.4	129.9	91.5	8.9	968.0	387.9
UF_pH10	1	9.5	198.3	128.0	8.6	747.4	284.2
UF_pH10	3	9.4	330.0	194.7	7.8	464.1	363.2
UF_pH10	24	9.2	406.2	412.2			
UF_pH11	0.5	10.1	25.5	24.0	8.8	502.2	120.6
UF_pH11	1	10.2	10.5	18.2	8.3	337.6	222.5
UF_pH11	3	10.0	42.6	36.8	7.5	158.4	97.7
UF_pH11	24	9.7	112.1	87.3			
UF_pH12	0.5	11.2	-4.5	20.7	7.5	36.7	5.2
UF_pH12	1	11.2	6.4	8.0	7.4	40.5	21.8
UF_pH12	3	11.0	5.2	8.8	7.6	16.4	6.7
UF_pH12	24	10.4	49.9	16.1			

Personal Message

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