Identification of the inactivating factors and mechanisms exerted on MS2 coliphage in concentrated synthetic urine

Wakana Oishi¹, Daisuke Sano^{2*}, Loic Decrey³, Syunsuke Kadoya¹, Tamar Kohn³, and Naoyuki Funamizu¹

¹Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, North 13, West 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

²Department of Civil and Environmental Engineering, Graduate School of Engineering, Tohoku University, Aoba 6-6-06, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan

³Laboratory of Environmental Chemistry, School of Architecture, Civil and Environmental Engineering (ENAC), École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

Corresponding author: Daisuke Sano, Ph.D.

Postal address: Aoba 6-6-06, Aramaki, Aoba-ku, Sendai, Miyagi 980-8579, Japan

E-mail: daisuke.sano.e1@tohoku.ac.jp

Tell: +81-22-795-7481

Abstract

Volume reduction (condensation) is a key for the practical usage of human urine as a fertilizer because it enables the saving of storage space and the reduction of transportation cost. However, concentrated urine may carry infectious disease risks resulting from human pathogens frequently present in excreta, though the survival of pathogens in concentrated urine is not well understood. In this study, the inactivation of MS2 coliphage, a surrogate for single-stranded RNA human enteric viruses, in concentrated synthetic urine was investigated. The infectious titer reduction of MS2 coliphage in synthetic urine samples was measured by plaque assay, and the reduction of genome copy number was monitored by reverse transcription-quantitative PCR (RTqPCR). Among chemical-physical conditions such as pH and osmotic pressure, uncharged ammonia was shown to be the predominant factor responsible for MS2 inactivation, independently of urine concentration level. The reduction rate of the viral genome number varied among genome regions, but the comprehensive reduction rate of six genome regions was well correlated with that of the infectious titer of MS2 coliphage. This indicates that genome degradation is the main mechanism driving loss of infectivity, and that RT-qPCR targeting the six genome regions can be used as a culture-independent assay for monitoring infectivity loss of the coliphage in urine. MS2 inactivation rate constants were well predicted by a model using ion composition and speciation in synthetic urine samples, which suggests that MS2 infectivity loss can be estimated solely based on the solution composition, temperature and pH, without explicitly

accounting for effects of osmotic pressure.

Keywords (max. 6):

Concentrated urine, culture-independent assay, human urine, urea hydrolysis, urine-diverting
toilet

1. INTRODUCTION

Human urine is rich in nitrogen, phosphate, and potassium, which are essential components for food production. Average amounts of these nutrients (phosphate, nitrogen, and potassium) in human urine are approximately 5.6 kg, 0.5 kg, and 1.0 kg per person per year, respectively, and account for 37%, 19%, and 54% of global consumption of fertilizer (Wolgast, 1995), respectively. However, human urine is commonly mixed with feces, which may pose infectious disease risks and impede safe use of human urine as a fertilizer (Karak et al., 2011). The introduction of a urine-diverting toilet is an effective approach for resource recovery from human excreta, but cross-contamination occurs (Schönning et al., 2002). A number of human pathogens, both viral and bacterial, could be detected in human urine collected from urine-diverting dry toilets (Bischel et al., 2015). The World Health Organization (WHO) reported that the storage of urine for longer than 6 months at temperatures higher than 20 °C in a closed tank achieved sufficient pathogen inactivation in urine in a large-scale system (WHO, 2006), but such long-term storage (more than a few months) may not be feasible for urine collection at a scale in dense urban environments due to storage volume requirements. The odor problem and the loss of recoverable nutrients by ammonia volatilization during long-term urine storage also have to be addressed.

Several treatment processes are available to tackle the limitations of urine storage (Maurer et al., 2006). Among them, volume reduction (condensation) is one of the options

which enables nutrient concentration, space saving and potential inactivation of pathogens. It can be achieved by evaporation, freeze-thaw, or reverse or forward osmosis (Maurer et al., 2006; Zhang et al., 2014). During and after the urine condensation process, several factors may contribute to inactivation. If volume reduction is achieved by evaporation, both temperature (Bertrand et al., 2012) and the evaporation process itself (Ward et al., 1977) can cause inactivation of microbes. Furthermore, if urine is hydrolyzed, condensation could lead to the concentration of uncharged ammonia (NH_3), a known biocidal compound (Warren, 1962; Cramer et al., 1983). And finally, urine condensation can enhance pathogen inactivation by increasing the osmotic pressure. It has been reported that the hyperosmolar conditions lead to the inactivation of pathogenic bacteria, because the raised concentration of ions, such as sodium and potassium ions, disturbs homeostasis inside the bacterial cells, and results in low enzyme activity and low penetration of metabolic products through the cell membrane (Kasumi, 2010). However, the inactivation of viruses during and after the urine condensation has not been investigated to date. For the safe use of nutrients in urine, it is important to correctly understand the inactivation profile of viruses during and after the urine condensation.

The aims of this study were therefore to evaluate the inactivation rate of MS2 coliphage, a surrogate of human enteric viruses, in concentrated urine, and to identify the relevant virucidal factors and mechanisms. First, the inactivation rate constants of MS2

coliphage in concentrated, non-hydrolyzed and hydrolyzed synthetic urine were evaluated and compared to those obtained in control buffer solutions. We then investigated the kinetics of genome loss by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using six different primer sets. Finally, the inactivation data were compared to those predicted by a model of MS2 inactivation established by Decrey et al. (2015). This model was previously developed and validated for urine and diluted urine. Here, we assessed if the same model can be used to predict inactivation in concentrated urine, or if additional processes not captured by the model influence inactivation if urine components are present in more concentrated form.

2. Materials and Methods

2.1 Synthetic urine samples

Synthetic urine was used instead of natural urine because of the ease to create and control the composition of concentrated solutions. Synthetic urine samples were prepared according to Griffith et al. (1976). There are three concentration levels: 1- (non-concentrated) and 5-, and 10-fold concentration. The concentrated synthetic urine was prepared by setting higher concentration of ingredients as shown in Table S1. The synthetic urine samples were poured into two 50-mL falcon tubes, and 6.5 mg urease from Jack Bean (Wako Pure Chemical Ltd.) were added to one of the bottles to hydrolyze the urea in the synthetic urine samples. Two days after preparation, the pH of the hydrolyzed synthetic urine samples was adjusted to

9.4 using 1N HCl or NaOH solution. Similar pH conditions were obtained in all experiments using hydrolysed synthetic urine in order to minimize effects related to pH variation. The pH values around 5.2 were always obtained for non-hydrolysed synthetic urine without pH adjustment.

2.2 Buffer solutions

Ammonium and carbonate buffer solutions were prepared to serve as NH_3 and pH/osmotic pressure controls, respectively. Ammonium chloride solutions and 25% (w/w) aqueous ammonia were blended to adjust their pH to 9.4. The composition of the ammonium buffer is shown in Table S2. Sodium carbonate buffer solution was prepared by blending sodium carbonate solution (0.05 M) and sodium hydrogen carbonate solution (0.1 M) at a ratio of 1:3 (The Chem. Soc. Japan, 1966), which results in a pH of 9.4. Sodium chloride was added to the carbonate buffer solution to vary osmotic pressure. The composition of the different carbonate buffers is shown in Table S2.

2.3 Measurement of total ammonium nitrogen and osmotic pressure

Total ammonia nitrogen (TAN: $\text{NH}_3 + \text{NH}_4^+$) and osmotic pressure were measured in a subset of samples. The TAN was measured by the indophenol blue method (Scheiner, 1976). One milliliter of a sample was diluted with deionized water to adjust the ammonia

concentration in the range from 0.1 mg-NH₄⁺/L to 5 mg-NH₄⁺/L prior to the measurement.

Osmotic pressure was measured by the freezing point depression method (Brunzel, 1994).

These corresponding values are reported in Table S3.

2.4 MS2 coliphage and *Escherichia coli*

MS2 coliphage (NBRC 102619) and *E. coli* (NBRC 13965) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). Firstly, *E. coli* was incubated for 5 hours in 10 mL of liquid broth, in a shaking water bath at 37 °C. The coliphage suspension (200 µL) was inoculated in the *E. coli* suspension and incubated overnight under the same conditions as above. The obtained suspension was centrifuged (3,000 ×g, 10 min) to separate the coliphage from the *E. coli* cells. The supernatant was passed through a membrane filter (0.20 µm pore, DISMIC®-25CS). The coliphage suspension was stored at -80 °C until use.

The double-layer method (Adams, 1959) was used to titer the infectivity of MS2 coliphage using *E. coli* (NBRC13965) as a bacterial host. The composition of top-agar and bottom-agar is shown in Table S4. Ten milliliters of bottom agar were poured into a petri dish and solidified at room temperature. One hundred micro liters of MS2 coliphage suspension were applied to the bottom-agar and mixed with 15 mL of top-agar, which contained the host *E. coli* (10⁸ colony forming units/mL). The samples were incubated at 37 °C overnight. Infective viruses were enumerated as plaque forming units (PFU) mL⁻¹, and results are

reported as the average of the plaque counts of triplicate plates. The detection limit was 300 PFU/mL.

2.5 Experimental setup

Thirty micro-liter of a virus solution containing 10^{10} - 10^{11} PFU mL⁻¹ was added to a 50-mL falcon tube containing 30 mL of experimental solution. There were four sample types; non-hydrolyzed synthetic urine, hydrolyzed synthetic urine, ammonium buffer and bicarbonate buffer. Varied osmotic pressure values between 720 and 9150 [mOsm/kg] were set in each sample type (Table S3) to see the effect of osmotic pressure and ingredients on the inactivation rate. The pH values of non-hydrolyzed synthetic urea were between 5.2 and 5.4, while those of the other three samples were set at 9.4, which was a measured value in hydrolyzed real urine in our preliminary experiment (data not shown). These samples with different ingredients but identical pH enable us to clarify the effect of pH on the virus inactivation rate. Experiments were carried out at constant temperature (20 °C). Incubation time of virus suspension in hydrolyzed synthetic urine was up to 24 hours, while that in non-hydrolyzed synthetic urine was up to 960 hours. Samples were taken and serially diluted in phosphate buffered saline (PBS) (5 mM PO₄³⁻, 10 mM NaCl, pH was adjusted to 7.5 with NaOH) and stored at 4 °C for no more than six hours prior to enumeration. In addition, samples were also taken and further processed (as described below) for RT-qPCR in the case

of non-concentrated and 10-fold concentrated hydrolyzed synthetic urine. Experiments were conducted in triplicate.

2.6 RT-qPCR for enumerating MS2 coliphage genome copies

Viral RNA was extracted using a QIAamp Viral RNA kit (Qiagen) according to the manufacturer's instructions. Extracted RNA was quantified by a one-step reverse transcription (RT)-qPCR using the 7500 Real Time PCR system (ABI). Each qPCR sample was run in 10 μL total volume comprising 5.0 μL of 2 $\times$ OneStep SYBR RT-PCR buffer III, 0.2 μL of TaKaRa ExTaq HS (5U/ μL), 0.2 μL of PrimeScript RT enzyme mix II, 0.2 μL of 10 μM forward primer, 0.2 μL of 10 μM reverse primer, 3.0 μL of nuclease free water, 0.2 μL of ROX Reference Dye II (50 $\times$ C), and 1 μL of RNA sample (TaKaRa Bio). We used six individual primer sets according to Wigginton et al. (2012). The following thermo-cycling conditions were used: 5 min at 42°C, 10 sec at 95°C, 40 cycles of 95°C for 5 sec, and 60°C for 34 sec.

2.7 Inactivation kinetics and damage to viral genome

Inactivation kinetics were determined by least-square fit of the data to a first-order model according to the following equation:

$$\ln \frac{C}{C_0} = -k_{obs}t \quad (1),$$

where C_0 and C [PFU mL⁻¹] are the virus concentrations at time 0 and t , and k_{obs} is the first-order inactivation rate constant [hr⁻¹]. Genome integrity was measured for the intact MS2 coliphage in hydrolyzed non-concentrated synthetic urine and 10-fold concentrated synthetic urine. Six genome segments, which jointly cover approximately 50 % of the total MS2 genome, were separately quantified by RT-qPCR. The decay of an individual segment was fitted to the first-order kinetic model. The intact proportion of the whole genome of MS2 coliphage was estimated using the following equation (Pecson et al., 2011):

$$N_t/N_0 = (\prod_i n_{it}/n_{i0})^{\text{Total genome length/Total length of six genome segments}} \quad (2),$$

where N_t/N_0 is the proportion of the whole genome that remains intact at time t , i is the genome segment number investigated at time t ($i = 1, 2, 3, 4, 5$, and 6), and n_{it}/n_{i0} is the measured proportion of each genome segment i . This equation does not assume that the damage rate is equal among genome segments (Pecson et al., 2011), but rather combines the damage rates of different genome regions to estimate the overall damage rate of the whole genome. The Welch's t test was performed to detect a statistically significant ($p < 0.5$) difference between log reduction values at each incubation time.

2.8 Model prediction and ammonia activity

Decrey et al. (2015) developed a model to estimate ion activities and to predict kinetic parameters for MS2 inactivation based on solution composition, pH and temperature (<https://lodecrey.shinyapps.io/MS2inactivation>). In short, MS2 inactivation rate constants are calculated as a function of the activity of bases (OH^- , NH_3 , CO_3^{2-} , HCO_3^- , PO_4^{3-} and HPO_4^{2-}) and their second-order inactivation rate constant for MS2. The base-specific, second-order rate constants were established empirically, and as a function of temperature (Decrey et al., 2015; Decrey and Kohn, 2017). The base activities are determined from the solution composition, pH and temperature using PHREEQC (Parkhurst and Appelo, 2013) and a database using the Pitzer approach. Inactivating effects of osmotic pressure are not explicitly considered. This model was used to assess NH_3 activities and predict the inactivation rate constants in the different solution tested herein (see Table S5 for the solution composition considered in the model). These predictions were then compared to the measured inactivation rate constants, to assess if factors beyond those considered in the model contribute to inactivation.

3. Results

3.1 Influence of urea hydrolysis and concentration of synthetic urine on MS2 inactivation

The inactivation of MS2 coliphage in non-hydrolyzed (Fig. 1A) and hydrolyzed synthetic urine (Fig. 1B) with three concentration factors (1-, 5-, and 10-fold) was investigated. In the non-hydrolyzed and non-concentrated synthetic urine, 960-hour incubation was insufficient to reach a 1- $\log_{10}$ reduction of the coliphage infectious titer, while 480- and 744-hour incubation was needed to reduce the coliphage infectious titer by 1- $\log_{10}$ in non-hydrolyzed and 5- and 10-fold concentrated synthetic urine samples, respectively (Fig. 1A). The inactivation rate in 5-fold concentrated and non-hydrolyzed urine was higher than that in 10-fold concentrated and non-hydrolyzed one ($p < 0.05$) with unknown reasons, but it is clear that the urine condensation without urea hydrolysis does not enhance virus inactivation. Meanwhile, the inactivation rate of MS2 coliphage was greatly enhanced when the synthetic urine sample was hydrolyzed (urease-treated) (Fig. 1B). In the hydrolyzed and non-concentrated synthetic urine, a 4- $\log_{10}$ reduction of the coliphage infectious titer was obtained after a 24-hour incubation period. In the hydrolyzed and 10-fold concentrated synthetic urine, a 6.8 $\log_{10}$ reduction was achieved after a 7-hour incubation. The inactivation rate constant was 2.28 per hour in the hydrolyzed and 10-fold concentrated synthetic urine, which was 5.9 times higher than that in hydrolyzed and non-concentrated synthetic urine ($p < 0.05$), and 729 times higher than that in non-hydrolyzed and 10-fold concentrated synthetic urine ($p < 0.05$) (Fig. 1C). These results indicate that the urine condensation can enhance the MS2 inactivation but urea hydrolysis needs to be associated.

3.2 Factors influencing MS2 inactivation in non-concentrated and concentrated synthetic urine

To identify the main physical-chemical virucidal factor in urine, the values of the inactivation rate constant were determined under varied conditions in terms of osmotic pressure, pH, and uncharged ammonia concentration (Fig. 2 and Table S3). There were no clear correlations between the inactivation rate constant and osmotic pressure (Fig. 2A) or pH (Fig. 2B). High inactivation rate constant were obtained when either the ammonium buffer or hydrolyzed synthetic urine were used (Fig. 2A), but this was not attributable to the higher osmotic pressure because the high osmotic pressure in the sodium carbonate buffer did not lead to the loss of MS2 infectivity. A higher pH value (9.4) resulted in higher inactivation rates than a lower pH value (5.2-5.6), but there was a significant variation at pH 9.4 (Fig. 2B). The inactivation rate constant in bicarbonate buffer (pH 9.4) was between 0.02 and 0.05 (h^{-1}), while those in ammonium buffer and hydrolyzed synthetic urine were always larger than 0.3 and reached 2.57 and 2.94 (h^{-1}) at maximum, respectively. Meanwhile, the rate constants were dependent on the uncharged ammonia activity (Fig. 2C). A linear approximation using all coupled datasets in Fig. 2C gave a determination coefficient value of 0.96. These results indicate that the uncharged ammonia is the most important factor for coliphage inactivation among the three factors analyzed.

3.3 Culture-independent estimation of MS2 infectivity based on genome amplification

The decay rate of six viral genome regions of RT-qPCR varied in both non-concentrated and 10-fold concentrated synthetic urine samples (Fig. 3A). Consistent with findings by Decrey et al. (2015), relatively homogeneous decay rates were observed for amplicons 2, 3 and 6. Amplicons 4 and 5, however, which were not explored by Decrey et al. (2016) exhibited variability. The highest value of the decay rate constant normalized by the amplicon length was obtained for region 4, in which 1.9×10^{-4} and 1.6×10^{-3} (h^{-1} per nucleotide) were obtained for non-concentrated and 10-fold concentrated synthetic urine samples, respectively. Meanwhile, the lowest value was obtained for region 5, with 1.8×10^{-5} and 1.3×10^{-5} (h^{-1} per nucleotide) for non-concentrated and 10-fold concentrated synthetic urine samples, respectively. There was 121-fold difference in the decay rate constant value (h^{-1} per nucleotide) between regions 4 and 5 for the 10-fold concentrated synthetic urine. We calculated the intact proportion of the whole genome of MS2 coliphage with equation (2) using all datasets of the six target regions. Equation (2) is based on the assumption that the overall damage rate across the genome segments analyzed and extrapolated to the full genome size, is equivalent to that of the whole genome, as described above. This assumption was validated because the intact proportion of the MS2 coliphage genome calculated by equation (2) was well correlated with the proportion of infectious MS2 coliphage in both non- and

10-fold concentrated synthetic urine samples (Fig. 3B). This positive correlation indicates that the intact proportion of the MS2 coliphage genome calculated by equation (2) using the six genome regions is applicable to the estimation of the infectivity loss of the coliphage in urine.

3.4 Model prediction of MS2 inactivation

Figure 4 shows a comparison between the inactivation rate constants predicted based on the model by Decrey et al. (2015), and the rate constants observed in this study. It is evident that the model provides an accurate prediction of MS2 inactivation rate constants, independently of osmotic pressure and ion concentrations, which were changed by synthetic urine condensation up to 10-fold. The consistency between predicted and observed inactivation rate constant serves as a confirmation that even in concentrated synthetic urine, MS2 infectivity loss can be estimated solely based on the solution composition, temperature and pH, without explicitly accounting for effects of osmotic pressure.

4. Discussion

4.1 Condensation of urine can enhance MS2 inactivation by concentrating biocidal substances

Volume reduction and hydrolysis of urine can potentially enhance pathogen reduction by concentrating harmful ions and introducing higher osmotic pressure conditions. A systematic investigation of MS2 inactivation was conducted in synthetic urine and different

buffer solutions that enabled us to disentangle the inactivating effects of three physical-chemical factors relevant to concentrated urine: ammonia activity, pH and osmotic pressure. The inactivation rate constants of the coliphage were well correlated with the activity of uncharged ammonia when compared to pH and osmotic pressure.

Uncharged ammonia is known to have a powerful biocidal effect (Warren, 1962; Cramer et al., 1983; Vinneras et al., 2008, Decrey et al., 2015), and it has been recognized as a sanitizer in human and animal waste (Fidjeland et al., 2013; Fidjeland et al., 2015). The results obtained in this study support that under the conditions investigated herein, uncharged ammonia is also the dominant sanitizing factor in hydrolyzed synthetic urine. In non-hydrolyzed synthetic urine, which lacks ammonia, MS2 inactivation is consequently very slow. Zuo et al. (2015) reported a protective effect of (non-hydrolyzed) concentrated urine constituents against common virucidal techniques (chlorine, UV and UV/TiO₂). It has been reported that osmotic pressure is one of the inactivating factors for viruses, but the susceptibility to salinity depends on virus type. Seo et al. (2012) showed that MS2 coliphage was persistent in solutions containing 0.3 – 6.3% NaCl, which approximately corresponds to 5 – 1100 mOsm/kg H₂O, whereas murine norovirus was more susceptible to the salinity. In the present study, osmotic pressure up to around 8000 mOsm/kg H₂O was shown not to be a causal factor for the inactivation of MS2 coliphage (Fig. 2A).

The experimental pH conditions of 5.2 and 9.4 were also not predominantly

responsible for the coliphage inactivation. The typical pH of fresh human urine is between 5.6 and 6.8 (Lind et al., 2000), but can rise to around 9 when urease produced by bacteria in feces decomposes urea into carbonate and ammonia. Feng et al. (2003) showed that the pH had to exceed 8.0 to induce MS2 inactivation. However, the sodium carbonate buffer at pH 9.4 did not reduce the infectious titer of the coliphage very rapidly in this study (Fig. 2B), which indicates that a pH value up to 9.4 is not the sole factor to explain the infectivity loss of the coliphage. Several studies have shown the effect of carbonate on bacteria (Park & Diez-Gonzalez, 2003) as well as on parasites (Fidjeland et al 2016), but under the conditions use in this study, carbonate was less effective at inactivating viruses. Vinneras et al. (2008) reported that coliphage inactivation decreased when stored urine was diluted without relevant pH changes. This supports our observations that inactivation in synthetic urine cannot be explained by pH effects alone.

4.2 Genome degradation is the mechanisms of MS2 inactivation independent of urine concentration

In this study, six regions in the whole genomic RNA of MS2 coliphage, which account for half of the whole length, were used in the culture-independent estimation of the infectivity loss of the coliphage. The six genome regions showed heterogeneous susceptibility (Fig. 3A), which may be caused by the complex secondary and/or tertiary structure of the

genomic RNA and the level of genomic association with viral structural proteins (Fiers et al., 1976; Fiers et al., 1975; Shiba and Suzuki, 1980). Even with the different susceptibility among the six genomic regions, the comprehensive decay profile measured by RT-qPCR was well correlated with the infectivity loss profile (Fig. 3B). As shown in the previous study (Pecson et al., 2011), the accuracy of this estimation increased with the increasing number of genome segments analyzed, but this study showed that the six genomic regions investigated, once extrapolated to the whole genome length, were enough to follow the inactivation profile of the coliphage in urine. Furthermore, the 1;1 relationship between infectivity loss and genome loss suggests that genome degradation alone can explain MS2 inactivation at all synthetic urine concentrations investigated (Fig. 3B). A previous study reported that the coliphage inactivation mechanism was RNA transesterification by base constituents in bulk solutions (Decrey et al., 2015). RNA ribose contains a hydroxyl group at the 2' position, which confers substantial instability to RNA compared to DNA deoxyribose. The presence of nucleophilic oxygen of the 2'-hydroxyl group close to the phosphodiester enables RNA cleavage by alkaline transesterification.

The correlation between genome decay rate estimated using the six target genome regions of RT-qPCR and the inactivation rate evaluated by the plaque assay is practically useful information because RT-qPCR for these amplicons can be available for estimating viral infectivity in the stored urine culture-independently. It may be worth establishing a

culture-independent assay for other human enteric viruses, such as human noroviruses, using RT-qPCR targeting multiple genome regions to analyze the inactivation profiles of human viruses in urine.

4.3. Model predictions of MS2 inactivation rates can be extended to concentrated synthetic urine

The model prediction of inactivation rate constants (Fig. 4) was valid independent of urine concentration, because osmotic pressure has no additional inactivating effect on MS2 (Fig. 2A) and the main inactivation mechanism was genome degradation by bases in solutions (Fig. 3B). The previous study by Decrey et al (2015) validated the model up to 150 mM NH_3 activity, while this study extended up to 1500 mM NH_3 and to complex solutions such as synthetic urine. This good agreement between the predicted and the observed inactivation rate constants further supports that alkaline transesterification is the main mechanism, under all conditions of urine concentration and osmotic pressure studied herein. However, these predictions would ultimately need to be confirmed in real concentrated urine, because additional factors such as microbial activity or presence of metal cations and organic compounds can influence MS2 inactivation in real urine. Furthermore, urine compounds such as metal cations or organic compounds, which might be harmless in non-concentrated urine, could potentially enhance or inhibit the inactivation mechanism shown in synthetic urine

when concentrating real urine.

4.4 Osmotic pressure remains a potential inactivating agent for other viruses and microorganisms

Even though MS2 inactivation was independent of osmotic pressure, this factor may nevertheless be relevant to the inactivation of other viruses. For example, murine norovirus was shown to be more susceptible to osmotic pressure than MS2 (Seo et al. 2012), which may suggest that animal viruses are more sensitive than phages to high salinity. Furthermore, DNA and double-stranded RNA viruses were shown to be resistant to inactivation by NH_3 (Decrey et al. 2016), which may increase the relative contribution of osmotic pressure to their inactivation. Therefore, the potential enhancing effect of osmotic pressure in the process of urine condensation needs to be explored further.

4.5 Anticipated challenges related to urine concentration and hygienisation

The present study showed that the condensation of urine is effective to control microbiological risks in urine utilization. The next challenge includes the optimization of the virus inactivation in urine. For example, high temperature is preferable for efficient virus inactivation, but the evaporation of ammonia, the most important virucidal factor, is enhanced at higher temperature. It is also not immediately evident whether the hydrolyzation of urine to

produce ammonia should be done before or after the urine condensation process. The optimization of urine condensation process will contribute to the safe management of virus infection risk in the utilization of nutrients in urine. We focused on the virus inactivation in the concentrated synthetic urine (after condensation conditions) in the present study, but the virus inactivation during the condensation process needs to be elaborated in the process optimization.

5. Conclusions

The inactivation rates were significantly faster in hydrolyzed synthetic urine than in non-hydrolyzed one. Uncharged ammonia was the leading factor for the inactivation in hydrolyzed synthetic urine, compared to osmotic pressure and pH under the investigated conditions. The six genomic regions are available for the culture-independent evaluation of infectivity loss of the coliphage in urine.

Acknowledgements

We really appreciate Dr. Ryusei Ito, Hokkaido University for his fruitful suggestions on this study. This work was supported by JSPS KAKENHI Grant Number JP15H02272. LD was supported by Swiss National Science Foundation Grant No. 200021_146829 / 1.

References

- Adams M. H. (1959). Bacteriophages. Interscience Publishers, New York, pp. 450-454.
- Bertrand I, Schijven J. F., Sánchez G., Wyn-Jones P., Ottoson J., Morin T., Muscillo M., Verani M., Nasser A., de Roda Husman A. M., Myrmel M., Sellwood J., Cook N. and Gantzer C. (2012). The impact of temperature on the inactivation of enteric viruses in food and water: a review. *J. Appl. Microbiol.*, 112(6), 1059-1074.
- Bischel H. N., Ozel Duygan B. D., Strande L, McArdell C. S., Udert K. M. and Kohn T. (2015). Pathogens and pharmaceuticals in source-separated urine in eThekweni, South Africa. *Water Res.*, 85, 57-65.
- Brunzel, N.A. (1994). Fundamentals of urine and body fluid analysis. W.B. Saunders, Philadelphia.
- Cramer, W. N., Burge, W. D. and Kawata, K. (1983). Kinetics of virus inactivation by ammonia. *Appl. Environ. Microbiol.*, 45, 760–5.
- Decrey L., Kazama S., Udert K. M. and Kohn T. (2015). Ammonia as an in-situ sanitizer: Inactivation kinetics and mechanisms of the ssRNA virus MS2 by NH₃. *Environ. Sci. Technol.*, 49, 1060-1067.
- Decrey L., Kazama S. and Kohn T. (2016). Ammonia as an in-situ sanitizer: Influence of virus genome type on inactivation. *Appl. Environ. Microbiol.*, 82(16), 4909-4920.
- Decrey L. and Kohn T. (2017). Virus inactivation in stored human urine, sludge and animal

manure under typical conditions of storage or mesophilic anaerobic digestion.

Environ. Sci. Water Res. Tech., accepted

Feng Y. Y., Ong S. L., Hu J. Y., Tan X. L. and Ng W. J. (2003). Effect of pH and temperature on the survival of coliphage MS2 and Q β . *J. Ind. Microbiol. Biotech.*, 30, 549-552.

Fidjeland J., Magri M. E., Jönsson H., Albiñ A. and Vinnerås B. (2013). The potential for self-sanitisation of faecal sludge by intrinsic ammonia. *Water Res.*, 47, 6014-6023.

Fidjeland J., Nordin A., Pecson B. M., Nelson K. L. and Vinneras B. (2015). Modeling the inactivation of ascaris eggs as a function of ammonia concentration and temperature. *Water Res.*, 83, 153-160.

Fidjeland J., Nordin A. and Vinneras B. (2016). Inactivation of Ascaris eggs and Salmonella spp. in fecal sludge by treatment with urea and ammonia solution. *J. Water Sanitation Hyg. Dev.*, 6(3), 465-473.

Fiers W., Contreras R., Duerink F., Haegeman G., Iserentant D., Merregaert J., Minjou W., Molemans F., Raeymaekers A., Vandenberghe A., Volckaert G. and Ysebaert M. (1976). Complete nucleotide sequence of bacteriophage MS2. *Nature*, 256, 274-278.

Fiers W., Contreras R., Duerink F., Haegeman G., Merregaert J., Minjou W., Molemans F., Raeymaekers A., Volckaert G., Ysebaert M., Vandekerckhove J., Nolf F. and Vanmontagu M. (1975). A-protein gene of bacteriophage MS2. *Nature*, 256, 273-278.

- Griffith D.P., Musher D.M. and Itin C. (1976). The primary cause of infection induced urinary stones. *Invest. Urol.*, 13(5), 347-350.
- Karak T. and Bhattacharyya P. (2011). Human urine as a source of alternative natural fertilizer in agriculture: A flight of fancy or an achievable reality. *Resour. Conserv. Recycl.*, 55, 400-408.
- Kasumi T. (2010). Polyol production as a compatible solute under hyperosmotic environmental conditions in yeast. *Journal of the Brewing Society of Japan*, 105(10), 618-627.
- Lind B., Ban Z. and Byden S. (2000). Nutrient recovery from human urine by struvite crystallization with ammonia adsorption on zeolite and wollastonite. *Bioresour. Technol.*, 73, 169-174.
- Maurer M. P. W., Pronk K. and Larsen T. A. (2006). Treatment process for source separated urine. *Water Res.*, 40(17), 3151-3166.
- Park G. W. and Diez-Gonzalez F. (2003). Utilization of carbonate and ammonia-based treatments to eliminate *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 from cattle manure. *J. Appl. Microbiol.*, 94, 675-685.
- Parkhurst D. L. and Appelo C. A. J. (2013). Description of input and examples for PHREEQC version 3 - A computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations: U.S. Geological Survey Techniques

and Methods, book 6, chap. A43, 497 p., available only at

<http://pubs.usgs.gov/tm/06/a43/>.

Pecson B. M., Ackermann M. and Kohn T. (2011). Framework for using quantitative PCR as a nonculture based method to estimate virus infectivity. *Environ. Sci. Technol.*, 45, 2257–2263.

Scheiner D. (1976). Determination of ammonia and Kjeldahl nitrogen by indophenol method. *Water Res.*, 10(1), 31-36.

Schönning C., Leeming R. and Stenström T. A. (2002). Faecal contamination of source-separated human urine based on the content of faecal sterols. *Water Res.*, 36(8), 1965-1972.

Seo K., Lee J. E., Lim M. Y. and Ko G. (2012). Effect of temperature, pH, and NaCl on the inactivation kinetics of murine norovirus. *J. Food Prot.*, 75, 533-540.

Shiba T. and Suzuki Y. (1980). Localization of A protein in the RNA-A protein complex of RNA phage MS2. *Biochim. Biophys. Acta*, 654, 249-255.

The Chemical Society of Japan. (1966). The chemistry handbook, Basic 2 (Kagakubinran Kisohe 2). Maruzen, Tokyo, Japan, pp. 1312-1320.

Vinneras, B., Nordin, A., Niwagaba, C. and Nyberg K. (2008). Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate. *Water Res.*, 42, 4067-4074.

- Ward R. L. and C S Ashley C. S. (1997). Inactivation of enteric viruses in wastewater sludge through dewatering by evaporation. *Appl. Environ. Microbiol.*, 34(5), 564-570.
- Warren K. S. (1962). Ammonia toxicity and pH. *Nature* 195, 47-49.
- WHO (2006). WHO Guidelines for the safe use of wastewater, excreta and greywater, Excreta and greywater use in agriculture, vol. 4., Geneva.
- Wigginton K. R., Pecson B. M., Sigstam T., Bosshard F. and Kohn T. (2012). Virus inactivation mechanism: Impact of disinfectants on virus function and structural integrity. *Environ. Sci. Technol.*, 46, 12069-12078.
- Wolgast, M. (1995). Stencil WM-Ekologen ABCo, Stockholm, Box 11162, S-10062 Stockholm, Sweden (In Swedish).
- Zhang J., She Q., Chang V. W. C., Tang C. Y. and Webster R. D. (2014). Mining nutrients (N, K, P) from urban source-separated urine by forward osmosis dewatering. *Environ. Sci. Technol.*, 48, 3386-3394.
- Zuo X., Chu X. Hu J. (2015). Effects of water matrix on virus inactivation using common virucidal techniques for condensate urine disinfection. *Chemosphere*, 136, 118-124.

Figure captions

Figure 1. Inactivation profile and rate of MS2 coliphage in synthetic urine. A) The time-course change in the concentration of infectious MS2 coliphage in non-hydrolyzed synthetic urine. B) The time-course change in the concentration of infectious MS2 coliphage in hydrolyzed synthetic urine. C) The inactivation rate constant values of MS2 coliphage in non-hydrolyzed and hydrolyzed synthetic urine samples. Error bars indicate the standard deviation of the mean. Asterisk indicates a statistically significant difference ($p < 0.05$) in t-test.

Figure 2. Inactivation rate constant values of MS2 coliphage in synthetic urine samples (non-hydrolyzed/hydrolyzed and 1-, 5- and 10-fold concentrated synthetic urine) and buffer solutions. A) The dependency of the inactivation rate on osmotic pressure. B) The dependency of the inactivation rate on pH, C) The dependency of the inactivation rate on the activity of uncharged ammonia.

Figure 3. Culture-independent assay for estimating the infectivity loss of MS2 coliphage in synthetic urine. A) Normalized inactivation rate constants (k_{norm}) in non-concentrated urine and 10-fold concentrated urine for each primer set. B) Relationship between infectivity loss

and genome number reduction of the coliphage. Error bars indicate the standard deviation of the mean of duplicate measurement. The solid line represents the 1:1 relation between infectivity loss and genome loss.

Figure 4. Comparison of observed and predicted MS2 inactivation rate constants in ammonia and carbonate buffer as well as in non-, 5-fold and 10-fold concentrated hydrolyzed synthetic urine. The solid line represents a 1:1 relation between measurement and prediction ($k_{pred}/k_{obs} = 1$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Graphical Abstract