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Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate

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ABSTRACT

Source separation and reuse of human urine can decrease the environmental pollution of recipient waters and reduce the need for artificial mineral fertilisers. However, the reuse of urine introduces another pathogen transmission route that needs to be managed. The inactivation of enteric pathogens and model organisms (*Salmonella enterica* subspecies 1 serovar Typhimurium (*S. typhimurium*), *Enterococcus faecalis*, bacteriophages *S. typhimurium* 28B, MS2 and $\Phi\times$ 174) by urine storage was studied at dilutions (urine:water) 1:0, 1:1 and 1:3 at temperatures 4, 14, 24 and 34 °C. A threshold concentration of ammonia was found at approximately 40 mM NH_3 (e.g. 2.1 g NH_3-N L^{-1} and pH 8.9 at 24 °C), below which all studied organisms, except *Salmonella*, persisted considerably longer irrespective of treatment temperature, showing that urine dilution rate is of great importance for pathogen inactivation. For *Salmonella* spp. no threshold level was found in these studies (15 mM NH_3 lowest concentration studied). At temperatures below 20 °C, bacteriophage reduction was very slow. Therefore, urine stored at temperatures below 20 °C carries a high risk of containing viable viruses. The study indicated that the current recommended storage time for urine of 6 months at 20 °C or higher is safe for unrestricted use and could probably be shortened, especially for undiluted urine.

1. Introduction

Separating human urine at source enhances the sustainability and efficiency of wastewater management. In domestic wastewater, the urine fraction contains most of the nutrients and only a minor proportion of the heavy metals and constitutes less than 1% of the volume, making it suitable for nutrient recycling (Vinnerås et al., 2006). Source separation of human urine for agricultural use is already being used in practice (Jönsson and Vinnerås, 2007; Mnkeni et al., 2008). The nutrient load on recipient waters is decreased as the majority of the nutrients, especially nitrogen, are redirected and can be used as a complete fertiliser applied as liquid manure (Kirchman and Pettersson, 1995; Fittschen and Hahn, 1998; Johansson, 1999). In terms of the nutrient loads used in Swedish crop production, human urine could replace 19, 20 and 29%, respectively, of the N, P and K applied in the form of artificial mineral fertiliser. Regarding the health risks when fertilising with human urine, transmission of infectious diseases by pathogenic microorganisms is the major concern.

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Only a few pathogens are normally excreted via the urine: *Schistosoma haematobium*, *Salmonella typhi*, *Salmonella paratyphi* and *Leptospira interrogans* (Feachem, 1983). In addition, *Mycobacterium tuberculosis* can be excreted in the urine of humans with renal TB infection (Daher et al., 2007). The main external source of potential pathogens in source-separated urine is misplaced faeces and on average the urine is contaminated with 9.1 mg faeces L^{-1} (Schönning et al., 2002). Urine from humans can contain pathogenic viruses (Vanchiere et al., 2005) but they are of minor concern for environmental transmission compared with the large number of enteric viruses that may enter the urine through faecal contamination in the sanitation system (Höglund et al., 2002). Introducing human-derived fertilisers from source-separating sewage systems may also introduce new routes of transmission for pathogens that need to be managed.

The generally recommended and only large-scale sanitation treatment available for urine is storage in closed tanks (Maurer et al., 2006). This sanitation method has been evaluated concerning inactivation of pathogenic microorganisms and indicator organisms at 4–5 °C and at 20 °C (Höglund, 2001). Based on these studies and a risk assessment, guidelines (Schönning and Stenström, 2004; WHO, 2006) recommend storage of collected urine for 6 months at 20 °C for a satisfactory reduction in microbial content covering a wide range of pathogenic bacteria (Höglund et al., 1998), some viral agents (Höglund et al., 2002) and *Cryptosporidium parvum* (Höglund and Stenström, 1999). Uncharged ammonia (NH_3) was the proposed agent for microbial inactivation but was not quantified in experiments.

The biocidal effect of uncharged ammonia is well known (Warren, 1962) but to have any substantial amount of ammonia in the uncharged state, an alkaline pH is required (Emerson et al., 1975). The main proportion of the nitrogen in urine is excreted as urea, which is transformed into ammonia during collection and which increases the pH to 8.8–9.2 in the collection tank (Vinnerås et al., 2006; Jönsson and Vinnerås, 2007). This means that storage can be an adequate treatment to sanitise source-separated urine. Further studies are needed with a wider range of temperatures and ammonia concentrations for more specific treatment recommendations in different regions and collection systems. This study investigated microbial reduction rates of bacteria and viruses in stored urine at different temperatures and different concentrations of uncharged ammonia.

The main objective was to improve the accuracy in predicting the efficiency of urine storage as a sanitation method depending on system and environmental factors. Inactivation of bacteria and bacteriophages was studied as a function of urine storage time and uncharged ammonia concentration. The uncharged ammonia concentration is regulated by temperature, pH and concentration of total ammonia. Three urine dilutions and four storage temperatures were investigated for up to 7 months in triplicate studies. The dilutions ranged from undiluted urine (6.0 g $N_{NH3/NH4}$ L^{-1}) through 1:1 dilution to 1:3 dilution (1.5 g $N_{NH3/NH4}$ L^{-1}), representing urine concentrations that can be found in practice in existing sanitation systems, and the temperature was set at 4, 14, 24 or 34 °C to cover a range of ambient storage temperatures.

2. Materials and methods

2.1. Materials

Urine collected from a source-separating low-flush sanitation system (pH 9.0 ± 0.01 and 3.2 ± 0.17 g $N_{NH3/NH4}$ L^{-1}) was used as the 1:1 dilution and urine collected fresh from three persons, two adults (male and female) and one toddler, was used for the other two degrees of dilution, 1:0 and 1:3. The fresh urine was treated with urease (Merck KGaA; Damstadt, Germany) added at a rate of 50 mg L^{-1} urine and left for 24 h at 37 °C for degradation of urea, which was confirmed by an increase in pH to 9.0. This urine was subsequently diluted in proportions 1:3 with tap water.

The bacteria used in the study were *Salmonella enterica* subspecies 1 serovar Typhimurium (*S. typhimurium*) phage type 178, isolated from Swedish sewage sludge (Sahlström et al., 2004) and *Enterococcus faecalis* (ATCC 29212). The bacteriophages used were dsDNA somatic *S. typhimurium* bacteriophage 28B (Lilleengen, 1948); ssRNA enterobacteria phage MS2 (ATCC 15597-B1) and ssDNA coliphage $\Phi\times$ 174 (ATCC 13706-B1).

2.2. Experimental set-up

The experiment was carried out in triplicate at the four temperatures (4, 14, 24 and 34 °C) and three different dilutions of urine and water (1:0, 1:1, 1:3). To avoid interference between bacteriophages 28B and MS2, which infect the same host strain used for phage detection, these two organisms had to be studied separately and subsequently two subgroups of organisms were created and studied in separate urine containers for each combination of temperature and dilution. *E. faecalis* and 28B phage constituted one subgroup and *S. typhimurium* together with $\Phi\times$ 174 and MS2 phages the other. In addition, ammonia-free controls (physiological saline solution, 0.8–0.9% NaCl) of all organisms except MS2 were kept at all four temperatures studied.

The urine was kept at each temperature for >2 h before bacteria and bacteriophages were inoculated (phage suspension 2 mL L^{-1} urine and bacterial solution 10 mL L^{-1} urine, resulting in initial concentrations of 10^6–10^8 colony forming units (cfu) or plaque forming units (pfu) mL^{-1} urine). Bacteria were cultured in nutrient broth (Oxoid; Sollentuna, Sweden) for 16 h and the phage suspension was prepared according to standard procedures (ISO10705-1, 1995).

The urine of each temperature and subgroup was divided into 3 aliquots, placed in 500-mL plastic flasks for dilution 1:1 and 50 mL centrifuge tubes for dilution 1:0 and 1:3, filled up to minimise headspace volume. When these tests were repeated all treatments were performed in 50-mL centrifuge tubes. The flasks and tubes were incubated at the respective temperature in darkness in the laboratory.

2.3. Analysis

The microorganism content of the urine was analysed at doubling intervals from day 0.5 until day 128 and then at the final sampling point, which varied from 150 days (5 months)

to 202 days (7 months) for the different samples. At sampling, 1 mL urine solution was taken from each replicate, serially diluted 10-fold with the two first dilutions in phosphate buffer (M15 pH 7.2, SVA) to neutralise the pH, and then the following in physiological saline solution.

Bacteria were detected by standard methods on xylose lysine desoxycholate (XLD) agar (Oxoid) containing 0.15% sodium novobiocin (37 °C, 24 h) for the enumeration of *S. typhimurium*. *Enterococcus* spp. were enumerated on Slanetz–Bartley (SlaBa) agar (Oxoid) (37 °C, 48 h) without differentiating the added *E. faecalis* from *Enterococcus* spp. originating from the urine.

The standard ISO10705-1 (1995) double-layer agar method (Adams, 1959) was used to enumerate *S. typhimurium* bacteriophage 28B, enterobacteria phage MS2 and coliphage $\Phi\times$ 174. The bacterial host strain *S. typhimurium* phage type 5, *S. typhimurium* WG 49 (ATCC 700730) and *E. coli* ATCC 1370 were used for enumeration of the respective bacteriophages.

For measurement of total ammonia ($NH_3 + NH_4^+$), 1 mL of urine solution was removed from each replicate and analysed spectrophotometrically on a Thermo Aquamate (Thermo Electron Ltd, Cambridge, UK) using the indophenol blue method (Merck; Whitehouse Station, NJ) according to the manufacturer's instructions. The concentration of uncharged ammonia in solution was calculated from the measured total ammonia, pH and temperature according to Emerson et al. (1975). The ammonia was measured on days 5, 53 and 98 in the 1:1 dilution and after 150 days in urine diluted 1:0 and 1:3.

For analysis of pH, 3 mL of urine solution from each replicate were transferred to a glass tube and allowed to adjust to room temperature. The pH was measured with an Inolab pH meter (WTW, Germany) on days 5, 53, 98 and 202 in the 1:1 dilution and on day 150 in urine diluted 1:0 and 1:3.

Organism inactivation was tested against the hypothesis of first order kinetics and the time for one decimal reduction (t_{90}) was derived from the decay coefficients. Means of unadjusted variables were tested using the Excel Student *t*-test with confidence levels reported in the results. Plots were made in MS Excel for Windows version XP (Microsoft Corporation, U.S.).

3. Results

3.1. Ammonia nitrogen and pH

A stable pH was observed in the urine, with little deviation between replicates. In urine diluted 1:1, where the pH was monitored more frequently than in the other two dilutions, pH was 8.8 on day 5, regardless of storage temperature and organism. This pH was 0.2 pH units lower than that of the stock urine. The pH remained at 8.8 until day 98, after which a decrease to 8.7 on day 202 was observed for urine stored at 24 °C (pH not examined for 34 °C). A similar pH difference was observed for the undiluted and 1:3 diluted urine (day 150), with somewhat higher pH for urine stored at 4 and 14 °C compared with storage at 24 and 34 °C (Table 1).

However, regarding total ammonia concentration no significant ($p > 0.05$) difference between storage temperatures was measured and thus means for all temperatures are presented in Table 1. The concentration of total ammonia in 1:1 dilution urine on day 98 (3.15 ± 0.25 g $N_{NH3/NH4}$ L^{-1}) did not differ significantly ($p > 0.05$) from that at the start (3.21 ± 0.17 g $N_{NH3/NH4}$ L^{-1}), indicating that no losses had occurred during that time. The freshly collected, undiluted urine had an ammonia concentration of 5.97 ± 0.32 g $N_{NH3/NH4}$ L^{-1}, confirming the approximate 1:1 degree of dilution in the sanitation system.

The different origin of the urine may explain the slightly lower pH in the 1:1 dilution collected in the sanitation system compared with the freshly collected urine diluted 1:3. However, parameters other than ammonia and pH were not monitored to determine any chemical alteration due to collection and storage in the sanitation system. The decrease in pH at the higher temperatures was not explained by ammonia losses. Concentrations of NH_3 calculated from temperature, measured ammonia and pH and means of replicates at each temperature and dilution are presented in Table 1.

3.2. Microbial inactivation

3.2.1. Reduction kinetics and derivation of t_{90}

The bacteria reduction followed first order kinetics with a good fit. Where non-detection (ND) limits (for bacteria 10 cfu and for phages 1 pfu mL^{-1} urine) gave higher inactivation rate, i.e. steeper slope on the inactivation function, the ND limit value was included in the regression data. In addition, concentration count in the batch-wise inoculated urine was used as start concentration for the *S. typhimurium* data to compensate for few detections encountered before ND levels were reached. The artificially derived ND values were excluded for estimation of variability, expressed as percentage standard deviation (S.D.) for the t_{90} in Table 2.

Reduction kinetics for two of the bacteriophages, MS2 and $\Phi\times$ 174, deviated from first order kinetics with initially fast reduction followed by a breakpoint, after which a slower reduction took place. This is illustrated in Fig. 1 by the reduction in MS2 phage in urine diluted 1:1 stored at 34 °C. In order to avoid overestimating the sanitation efficiency in the initially fast reduction that may be due to artefacts such as adhesion to particles in the urine, this phase was excluded from the data set when fitting the function of inactivation, i.e. deriving t_{90} (Table 2).

3.2.2. Reduction rates of bacteria and bacteriophages

The bacteria studied were more sensitive to urine storage than the bacteriophages and the less the urine was diluted, the higher the inactivation rate. In the ammonia-free temperature controls, inactivation was slow for all organisms at all temperatures (Table 2) and for the bacteria some growth occurred during the first 2 weeks. In total no or small reduction was detected during 5 months for *Salmonella* spp. in any of the temperatures investigated.

The reduction in *Enterococcus* spp. was much slower than the reduction in *S. typhimurium*, especially when the urine was diluted (Table 2). The reduction in *S. typhimurium* was not related strongly to different urine dilutions, as t_{90} was not affected by the 1:1 and 1:3 dilutions and was at the most 6.5 days (Table 2). As regards the other organisms, there were inconsistencies for the results at 4 and 14 °C in that the

Table 1 – Concentration of measured total ammonia/ammonium nitrogen (g $N_{NH3/NH4}$ L^{-1} urine) as mean ± S.D. for the urine dilutions 1:0 (day 150), 1:1 (day 98) and 1:3 (day 150) for all storage temperatures; and pH and NH_3 (mM) for the dilutions 1:1 (day 202) 1:0 and 1:3 (day 150) stored at temperatures 4, 14, 24 and 34 °C

Dilution	$N_{NH3/NH4}$ (g L^{-1})	Storage temperature							
		4 °C		14 °C		24 °C		34 °C	
		pH	NH_3 (mM)	pH	NH_3 (mM)	pH	NH_3 (mM)	pH	NH_3 (mM)
1:0	5.97 ± 0.32	9.1	57 ± 6	9.1	109 ± 3	9.0	156 ± 11	9.0	232 ± 8
1:1	3.15 ± 0.25	8.8	16 ± 1	8.8	33 ± 3	8.8–8.7	60 ± 4	8.8[b]	95 ± 31
1:3	1.54 ± 0.18[a]	9.1	15 ± 1	9.0	24 ± 6	8.7	24 ± 6	8.7	40 ± 8

NH_3 concentrations were calculated from pH, temperature and $N_{NH3/NH4}$ concentrations.
a Two samples at 1:3 dilution deviated widely and were excluded from the calculation of the mean ($n = 22$).
b The pH in urine stored at 34 °C is from day 98.

reduction time in the temperature control was 5–100 times longer than in the urine.

Reduction in *Enterococcus* spp. at 34 °C was fast regardless of urine concentration, whereas at lower temperatures the concentrated urine resulted in significantly shorter t_{90} than the diluted urine. For undiluted urine the t_{90} was below 7 days at all temperatures. In the temperature control no or slow reduction was observed during 40 days of study at 24 °C and below, while at 34 °C only a small reduction was detected during that time.

For the bacteriophage *S. typhimurium* 28B phage (double-stranded DNA), no reduction was observed at 4 °C over 6 months of study, though there was inactivation at 24 and 34 °C in the same range as that of bacteriophages $\Phi\times$ 174 (single-stranded DNA) and MS2 (single-stranded RNA) with shorter time for the undiluted urine, t_{90} at most 15 days. At 4 and 14 °C, t_{90} for $\Phi\times$ 174 and MS2 ranged from 28 to 240 days, with little correlation to temperature and urine dilution, and no difference in sensitivity between the two phages was observed (Table 2).

4. Discussion

4.1. *Effects of temperature and NH_3 on reduction rates*

According to the hypothesis that the inactivation is correlated to the concentration of uncharged ammonia, the t_{90} for the organisms was plotted against temperature and concentration of NH_3 for each replicate. The slightly alkaline pH in the narrow range 8.7–9.1 made it possible to exclude single inactivation effects from pH. The plots revealed temperature and NH_3 dependence for inactivation of the organisms and also indicated threshold temperatures and NH_3 concentrations (Fig. 2).

At 34 °C the reduction rates in the organisms investigated showed a linear correlation with NH_3 concentration (Fig. 2a). At 24 °C, for all organisms investigated except *S. typhimurium*, a threshold concentration could be observed at approximately 40 mM, giving disproportionately longer t_{90} compared with higher NH_3 concentrations (Fig. 2b). Ammonia-free controls

Table 2 – Decimal reduction (t_{90}) times in days ± S.D. (%) for the bacteria *S. typhimurium* and *E. faecalis* and the bacteriophages MS2, $\Phi\times$ 174 and 28B at different temperatures and urine concentrations

Temperature (°C)	Concentrated urine:H_2O	*S. typhimurium*[a]	*E. faecalis*	MS2	$\Phi\times$ 174	28B
34	1:0	<0.1	<1.1 ± 16	<1.6 ± 19	<5.7 ± 36	1.5 ± 3[b]
34	1:1	0.2 ± 3	<1.2 ± 3	6.9 ± 10	<5.1 ± 5[b]	<15 ± 2
34	1:3	<0.3 ± 11	<3.0 ± 38	8.4 ± 18	13 ± 34	14 ± 49[b]
37	0:1	24[b]	16[b]	–	10[b]	65[b]
24	1:0	0.6 ± 18	2.3 ± 13	15 ± 3	12 ± 10	12 ± 19
24	1:1	2.1 ± 5	9.1 ± 5	25 ± 22	16 ± 3	51 ± 6
24	1:3	1.0 ± 53	47 ± 93	82 ± 9	71 ± 8	59 ± 37
24		31	NR	–	22	80
14	1:0	<1.2 ± 5	6.4 ± 100	71 ± 13	79 ± 6	18 ± 7
14	1:1	<5.3 ± 7	21 ± 2	89 ± 24	130 ± 21	NR
14	1:3	2.3 ± 6	28 ± 4	200 ± 20	100 ± 9	169 ± 58
14		33	NR	–	NR	NR
4	0:1	2.1 ± 9	6.3 ± 20	160 ± 8	120 ± 7	NR
4	1:1	6.5 ± 9	42 ± 9	73 ± 7	28 ± 9	NR
4	1:3	5.0 ± 7	33 ± 61	240 ± 44	150 ± 15	NR
4	0:1	NR	NR	–	NR	NR

NR = no reduction detected during 182 days, except for the temperature control (0:1) that was studied during 40 days.
a For *Salmonella* an initial concentration from the batch-wise contaminated urine was included in the function fitting as reduction took place rapidly.
b Performed in 37 °C.

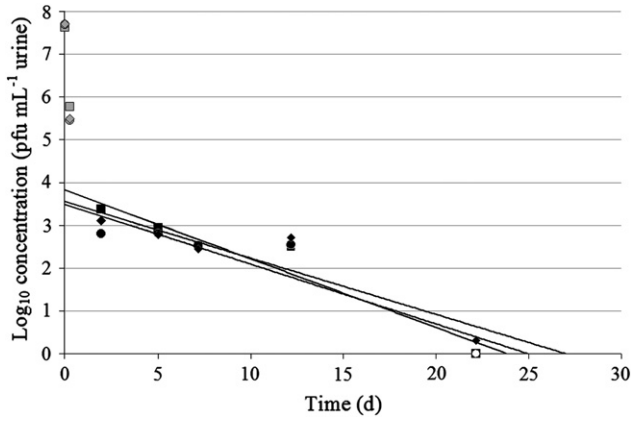

Fig. 1 – Log concentration (pfu mL^{-1}) of bacteriophage MS2, studied in triplicate, as a function of time (days) in urine diluted 1:1 at 34 °C showing the breakpoint between two sets of inactivation rates and the regression of an exponential function based on the latter rate. Samples excluded from the regression are marked with grey and ND values indicated with not filled symbols.

showed stable organism concentrations for 4 weeks, and at 24 and 34 °C both types of bacteria increased in numbers during the first 2 weeks (data not shown). This confirmed that the temperature works synergistically with NH_3 when threshold concentrations are reached.

At lower temperatures, 4 and 14 °C, the difference in reduction rates between bacteriophages and bacteria was large (Fig. 2c, d). A shift in inactivation rates exceeding 40 mM NH_3 could be seen, but not as clearly as for 24 °C (Fig. 2b). The major difference compared with the higher temperatures, even at NH_3 concentrations >50 mM, was that the reduction in bacteriophages was slower and also that the inactivation rate was less correlated to NH_3 concentration. In general, a doubling in biological activity is expected for every 10 °C increase in temperature. In this study the inactivation rates for the same urine dilution changed more than this in the temperature span between 14 and 24 °C. This can partly be explained by the larger proportion of NH_3 at higher temperatures, but mainly by synergistic effects from temperature and ammonia.

4.2. *Reduction in* S. typhimurium *and* Enterococcus *spp. in urine*

In addition to the reduction in *S. typhimurium*, the reduction in *Escherichia coli* O157 (CCUG 44857) was studied in 1:1 dilution urine at 4 and 34 °C. The *E. coli* was more sensitive to ammonia than the *S. typhimurium* strain used (data not shown). This is in agreement with results from ammonia treatment of *S. typhimurium* and *E. coli* O157 in sewage sludge (Mendez et al., 2004). In the present study, no breakpoint in the inactivation of *S. typhimurium* correlated to NH_3 concentration was found, with the lowest concentration investigated 15 mM. This is in line with Park and Diez-Gonzalez (2003), who found 5 mM NH_3 to be the lowest inhibitory concentration for *S. typhimurium* and *E. coli* studied in broth solution.

At ammonia concentrations ≥40 mM (i.e. at 34 °C for all dilutions and at all temperatures for undiluted urine), the reduction in *Enterococcus* was rapid ($t_{90} < 10$ days) but at lower concentrations the reduction rate decreased. *E. faecalis* as indicator organism for the reduction of *Enterobacteriaceae* such as *Salmonella* spp. and *E. coli* O157 is very conservative, especially at low ammonia concentrations, i.e.<40 mM NH_3, where the difference in t_{90} was almost 10-fold. In addition, the times for inactivation of the two groups of organisms, *Salmonella* and *Enterococcus*, did not correlate with each other ($R^2 = 0.4$).

Even if *Enterococcus* spp. seemed to behave similarly to the phages investigated (Fig. 2), *Enterococcus* is not an appropriate indicator for the inactivation since when the t_{90} were compared at different dilutions and temperatures, no correlation was found between the organisms ($R^2 < 0.3$).

4.3. *Reduction in bacteriophages in urine*

The *S. typhimurium* phage 28B was the most resistant of the organisms investigated and at the lower temperatures (4 and 14 °C) little or no reduction at all was noted. The inactivation of *S. typhimurium* phage 28B in urine has also been studied by Höglund et al. (2002) at temperatures 5 and 20 °C. The urine in that study had approximately 3.3 g $N_{NH3/NH4}$ L^{-1} and pH 9, giving 60–70 mM NH_3 at 20 °C, which resulted in t_{90} of 71 days. In the present study the 1:1 diluted urine stored at 24 °C with 51 mM NH_3 was the most similarly effective treatment, resulting in somewhat faster reduction with t_{90} of 51 days. At 5 °C little reduction took place in 200 days according to Höglund et al. (2002), results analogous to those in the present study at 4 °C. Höglund et al. (2002) compared the inactivation time with that of rhesus rotavirus (dsRNA), which was considerably shorter than for *S. typhimurium* phage 28B, although no significant difference was observed between urine and controls at either 4 °C or 20 °C. Positive relationships between inactivation rates of rotavirus and increased temperature have been found by Moe and Shirley (1982) in studies on faecal samples. Inactivation of bacteriophage 28B in faecal matter (19% DM) at 20 °C has also been studied by Vinnerås et al. (2003), where addition of 3% urea nitrogen gave >7 log_{10} reduction in 50 days whereas concentrations in the faecal control remained stable.

The MS2 phage (ssRNA) was more affected than $\Phi\times$ 174 (ssDNA) at 34 °C, whereas at the lower temperatures studied the two phages were equally affected by the ammonia treatment. In the reduction that took place at 4 and 14 °C, the inactivation was less correlated to treatment factors and prediction of treatment efficiency may therefore be difficult. For 28B little or no reduction was detected throughout 182 days of study (Table 2).

Ammonia solution has been confirmed to be virucidal for several polioviruses (type 1:CHAT and Mahoney; type 2: strain 712) and other enteric viruses (coxsackie A13 and B1, Echovirus 11, reovirus type 3) at 21 °C (Ward and Ashley, 1977; Ward, 1978). In those studies, the reduction at pH 9.5 (290 mM NH_3) was fast for all viruses (>5 log_{10} reduction in 24 h) except for the reovirus (dsRNA), which was less sensitive to the treatment (~2 log_{10} in 24 h). The concentration of uncharged ammonia used (290 mM) was not reached in the present study, where the highest concentration (in 1:0 urine at 34 °C) was 230 mM.

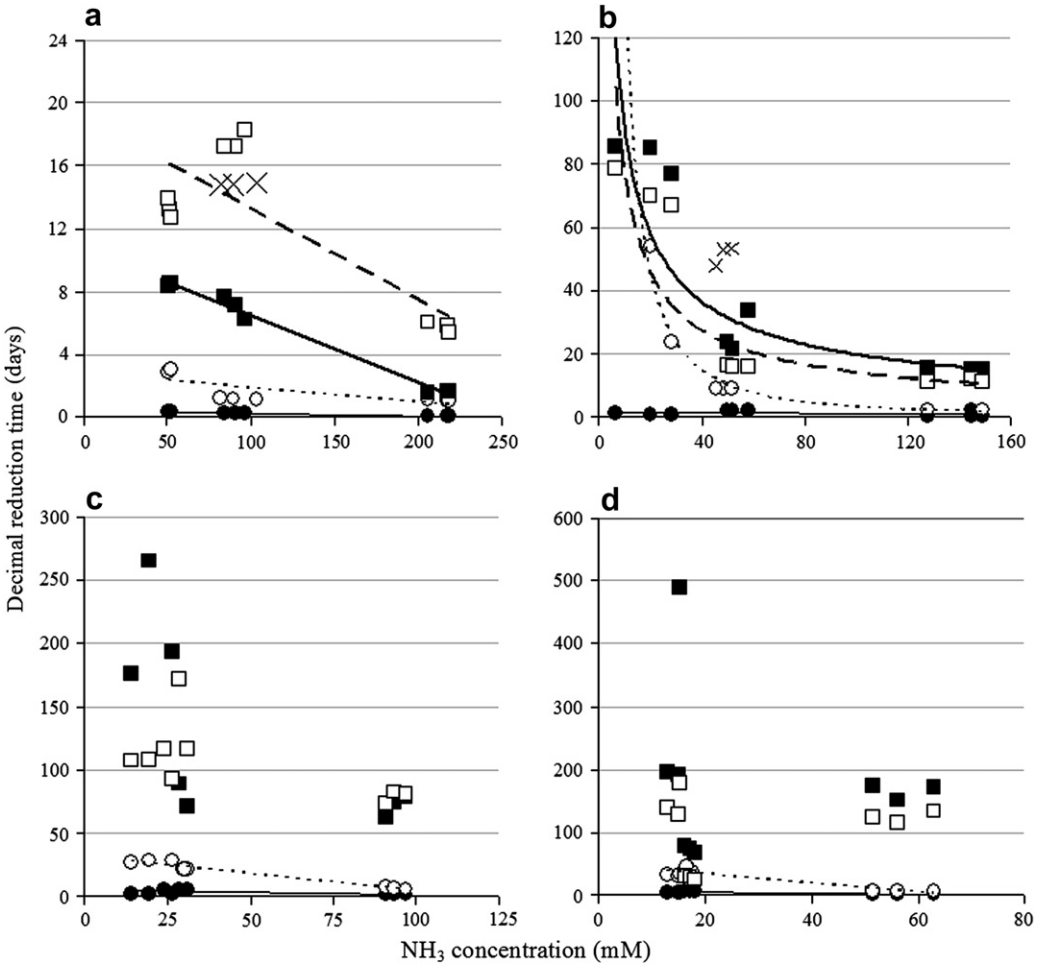

Fig. 2 – Time (days) for one decimal reduction (t_{90}) plotted as a function of concentration of uncharged ammonia (NH_3 (aq), mM) for *S. typhimurium* (●), *Enterococcus faecalis* (○), and the bacteriophages MS2 (■), *Φ*× 174 (□) and 28 B (×) in urine stored at temperatures (a) 34, (b) 24, (c) 14 and (d) 4 °C. The correlation trend is indicated with thin lines for the bacteria (*S. typhimurium* broken line) and with bold lines for the phages (*Φ*× 174 broken).

However, the sensitivity of the enteric viruses indicates that they may be reduced faster than the phages tested here. Studies of the ammonia sensitivity of coated ssRNA viruses, e.g. Avian influenza, indicate a much faster reduction for coated viruses compared with the reduction in the phages used in the present study (Emmoth et al., 2007).

The proposed inactivation mechanism for ssRNA viruses is cleavage of the RNA in intact particles with otherwise little structural alteration (Ward, 1978). The impact of ammonia on poliovirus and bacteriophage f2, the structure of which are comparable to that of MS2, was examined by Cramer et al. (1983) at 20 °C. Both organisms showed a log-linear correlation between NH_3 (2–180 mM) and k (corresponding to t_{90} 1–90 days) despite varying pH in the range 7–9 and the relationships being parallel, with polio having a 4.5 faster inactivation than bacteriophage f2.

4.4. *Influence of dilution and temperature on storage time*

At 34 °C the reduction in the organisms investigated was fast and the reduction in the otherwise stable bacteriophages, with t_{90} of just over 2 weeks, indicates that storage for more than 2 months should not be necessary. However, this needs to be confirmed with studies on reduction rates of animal viruses. The most interesting animal viruses are the double-stranded RNA viruses, e.g. rotavirus, and the double-stranded DNA viruses, e.g. adenoviruses, as the single-stranded viruses seem to be more sensitive to ammonia than the phages (Cramer et al., 1983).

At 24 °C a slower reduction was observed for the phages and for *Enterococcus* spp. when the urine was diluted 1:3 with water, giving concentrations of $NH_3 < 40$ mM. The threshold of 40 mM NH_3 seems to be accurate for all the organisms investigated (except for *Salmonella* spp. where the threshold is much lower) and for ascaris (Nordin et al., 2008). This indicates that for safe use of urine after 6 months of storage at 20 °C, it also needs to have an ammonia content above 40 mM, e.g. 2.8 g $N_{NH3/NH4}$ L^{-1} and pH 8.8. At higher pH or temperature, a somewhat lower ammonia concentration would result in the same concentration of NH_3. However, when fulfilling this requirement it seems that the required storage time could probably be shortened, especially for samples with high ammonia content and at temperatures well above 20 °C.

At temperatures below 20 °C, no assurance can be given that urine is safe regarding content of parasites, based on studies of *Ascaris suum* (Pecson and Nelson, 2005; Pecson et al., 2007; Nordin et al., 2008), *C. parvum* and viruses (Jenkins et al., 1998; Höglund and Stenström, 1999) including phages (Höglund et al., 2002), as the reduction in these organisms is slow at low temperatures. The slower reduction is a combination of less NH_3 (Table 1) and a lower cell permeability at lower temperatures (Jenkins et al., 1998). However, at ammonia levels that correspond to concentrated urine (6 g $N_{NH3/NH4}$ L^{-1}, i.e. >40 mM NH_3), the t_{90} for both *Salmonella* spp. (Table 1) and *C. parvum* (Jenkins et al., 1998) is less than 2 weeks, even at 4 °C. As these are two major zoonotic risk organisms in urine, restricted use to crops not intended for human consumption can be accepted after only 2 months of storage even at temperatures as low as 4 °C and having a reduction corresponding to >4 log_{10}, while for lower ammonia concentrations longer storage time is needed.

5. Conclusions

The dilution rate is an important factor regarding the reduction in pathogenic microorganisms in urine, especially at temperatures ≤24 °C, where low ammonia concentrations result in slow inactivation. A threshold for inactivation was identified at approximately 40 mM NH_3, below which inactivation was slow. This concentration was met at all temperatures when urine was undiluted. The NH_3 is regulated by total ammonia concentration, temperature and pH.

At temperatures below 20 °C, there ought to be restrictions on the use of urine as a fertiliser on food crops, as ascaris and viruses are reduced at a very slow rate. Zoonotic organisms, e.g. *Salmonella* spp. and *C. parvum*, which restrict other uses as a fertiliser, e.g. on fodder crops, are reduced by >4 log_{10} within 2 months at temperatures from 4 °C if the uncharged ammonia concentration is above 40 mM. Lower concentrations need longer storage time.

At 34 °C, where the concentration was above 40 mM even when urine was diluted 1:3, fast inactivation of all organisms was observed. However, without ammonia present the survival of pathogens at this temperature was high.

For safe, unrestricted, reuse of urine fulfilling the requirement of 40 mM uncharged ammonia above 20 °C, the required storage time according to WHO guidelines could probably be shortened, especially for samples with high ammonia content and at temperatures well above 20 °C.

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