Use of faecal pollution indicators to estimate pathogen die off conditions in source separated faeces in Kathmandu Valley, Nepal

Anjali Manandhar Sherpa, Denis Byamukama, Roshan R. Shrestha, Raimund Haberl, Robert L. Mach and Andreas H. Farnleitner

ABSTRACT

As the introduction and promotion of dehydrating toilets progresses, the safety of handling and reuse of their biosolids remains a question. A detailed study to understand the storage conditions and the fate of selected faecal indicators was conducted on four urine diverting dehydrating toilet units, using ash as a major additive, in Kathmandu Valley, Nepal. Presumptive *Escherichia coli*, total coliforms, enterococci and different fractions of *Clostridium perfringens* were investigated under field storage conditions. In addition, chemo-physical and chemical (carbon, nitrogen, phosphorous content) parameters were investigated. Observed temperature was low in all the four toilets with a median of 24.0°C, which was in the same range as the ambient temperature. pH was below the desired range of >9 and moisture level was very high (>60%). No single factor of the studied chemo-physical and chemical parameters could be found by statistical analysis to have accounted for the reduction of the indicators in any of the toilets. By time series analysis of the investigated strata in the faecal heaps ($n = 96$), the determined reduction rate showed increasing persistence characteristics for *E. coli*, coliforms and enterococci with respective average $\log_{10}$ reduction of -0.4, -0.3 and -0.2 per month ($p < 0.001$). No significant reduction was observed for the different fractions of *C. perfringens* determined for the non-pasteurised and pasteurised fraction at 60°C and 85°C. 72% of randomly selected and analysed samples ($n = 36$) were found to contain helminthes eggs. The used 6 months storage time did not prove sufficient to reach appropriate safety levels for handling and reuse of the biosolids.

Key words | chemo-physical and chemical factors, indicator microorganisms, microbial die-off, sanitation, urine, urine diverting dehydrating toilets

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INTRODUCTION

Access to sanitation remains a big challenge. According to the Millennium Development Goals as many as 1.9 billion people will need to gain access to improved sanitation by 2015 in order to reach the proposed targets (WHO 2006). In Nepal, water and sanitation issues have just recently received considerable attention (Shrestha & Sharma 2001). There has been a substantial increase in water supply coverage, from a mere 4% in 1970 to 78% of rural and 92% of the urban population in 2000 (Shrestha 2003). Access to sanitation has however, not shown much improvement in the country. In many peri-urban communities in Kathmandu Valley, water and sanitation facilities are still lacking and people use septic tanks and pit latrines which are poorly constructed and are a pollution risk to ground water, especially given the high water table.

doi: 10.2166/wh.2009.149

Given this situation, in 2002, urine diverting dehydrating toilets were introduced in Nepal as a viable sanitation alternative and possible source of organic fertilisers for sustainable agriculture. Currently more than 450 urine diverting dehydrating toilets are in operation in different peri-urban farming communities in Kathmandu Valley implemented by different NGOs. Most of the toilets are operated with a storage time of 6 months.

One of the major concerns however, is the safety of the reuse of human faeces due to the presence of potential pathogens. Faeces contain roughly around 10^{12} commensalistic enteric microorganisms per gm, with faecal coliform bacteria in the range of 10^6–10^9 CFU per gm (WHO 2006). In the case of infections, however, pathogenic viruses, bacteria, parasitic protozoa and helminthes can be excreted in very high amounts with human faeces (Esrey *et al.* 1998; Fane 2004). Even though protozoa and viruses cannot grow in the environment, bacteria may increase due to re-growth (Höglund 2001). Usually, concentrations of enteric microbes decline by death or by loss of viability once outside the host body although bacteria, viruses, protozoa and helminthes eggs can survive for several months, and even years in the case of Ascaris eggs (Esrey *et al.* 1998; Esrey 2001).

Thus, handling and application of faecal matter in the agricultural lands without adequate treatment can lead to further transmission of pathogens in the environment and spread of diseases (Schönning & Stenström 2004). In urine diverting dehydrating toilets, various environmental factors, i.e. pH, temperature, moisture, nutrients and sunlight are important in accelerating the rate of microbial die off (Esrey *et al.* 1998; Höglund 2001) with varying degrees of impacts on different microorganisms (Schönning & Stenström 2004). Therefore, the conditions existing in the dehydrating toilets and the duration of storage are important factors determining the fate of the microbes. However, limited information is available on the dynamics of the environment in these systems during storage and the fate of the intestinal microbes. A four months' detailed study was conducted from May to August 2004 on four toilets at different storage stages. The study aimed to investigate the storage conditions in the dehydrating toilets and their effect on the survival of indicator microorganisms of differing persistence, namely *E. coli*, total coliforms (TC), enterococci and *C. perfringens* subjected to different pasteurisation regimes. The collected faecal samples from the toilets and respective layers in the storage vaults covered a total storage period of approximately one year.

MATERIALS AND METHODS

Study area

Khokana is a small farming community inhabited by indigenous people of the Valley–the Newars. It is located about 2 km south of the Nepalese Capital in Lalitpur District at an altitude of around 1,300 metres above sea level. There are a total of 818 households with a population of 4,542. In the past, farmers used human excreta as fertilizer but in recent years most of the farmers have started using chemical fertilizers. However, urine diverting dehydrating toilets have gained popularity among the farmers due to their willingness to revert to the old practise but in a more hygienic and safer manner. For the purpose of this study, three toilets were randomly selected from this community and the fourth toilet was located in a modern house situated in the urban settlement of Kathmandu.

Toilet design

Toilets (so called "ECOSAN" toilets) at Khokana were squatting type double-vault, urine diverting dehydrating toilets. Urine was diverted through a pipe to a collection tank and the faeces dropped into a vault where it was collected and stored. Each vault had a capacity of about 0.5 m^3 designed for a family of 5–6 persons for a storage period of approx. 6 months. Vent pipes were installed to remove any odour and gases produced and to facilitate air circulation. The urine collection tank of 100 litres capacity was a brick and mortar structure with a concrete lid and a tap at the base. A separate anal cleaning place was provided between the two pans and the wastewater was treated in a small constructed wetland. The toilet in the urban setting was a commode type urine diverting dehydrating toilet. Here, faeces and urine was collected in plastic containers. Toilet paper was used instead of water for anal cleaning. Ash was added after each visit in all the four toilets to cover the fresh faeces to make it invisible for the next user, to

reduce odour and to minimize flies. Water was poured in the urine bowl after every use to clean it and to reduce odour in both types of the toilets.

Sample collection and calculation of storage time

The study was conducted over a period of four months starting May 2004. The three toilet units from Khokana (A, B, D) had been used for 6 months and were closed in mid January, end of February and mid March 2004 respectively. Similarly, the toilet from the urban setting (C) had been used for 4 months and closed mid April 2004. Each of the four investigated toilets were sampled eight times during the study period of 4 months. Samples were collected and analysed individually from the top (TP), middle (MD) and bottom layers (BT) of each faecal heap. A total of 96 samples were analysed during the study period. The height of the faecal heaps of three toilets (A, B, D) was measured during every sampling to correct for the sampling heights especially for the middle layer as the height was expected to decrease with time due to compaction of faeces. Effect of physical compaction was taken into consideration and the height of the heap was calculated as a function of time. With this approach, the bottom of the heap would have the longest storage time and the top of the heap the lowest. This logic was followed for calculating the storage time of the respective faeces layers in the four units studied. The total storage time was calculated as the sum from i) respective storage time during use of the toilet as derived from the exact location of the investigated layer (i.e. TP, MD, BT), and, ii) storage time after closing the vault. For MD it was assumed that this layer was reached about half the time of use of the vault. The resulting total investigated storage time covered a period of approximately one year.

Analysed parameters

Chemo-physical parameters of temperature, moisture and pH were measured in this study. Temperature reading was taken at the time of sample collection for each layer using a digital temperature probe (S–631 07, Thermometerfabriken Viking ab Eskilstuna, Sweden). Wet weight was measured for a constant volume of 6 cm^3 of the sample for conversion of faecal mass into volume and expressing the counts of microorganisms as CFU/cm^3. Wet weight of the known volume of samples was taken before drying the sample in an oven at 105°C till constancy of the weight. The dried samples were cooled in a desiccator and the dry weight was taken. Moisture content was calculated as the percent difference of dry and wet weight for each sample (Eaton *et al.* 1995). To measure the pH, 10 gm of wet sample was mixed with 10 ml of distilled water, thoroughly mixed by shaking and allowed to stand for one hour. The pH of the supernatant was measured with a WTW pH meter (Wissenschaftliche Technische Werkstätten GmbH, WTW, Germany).

Nutrient parameters

Organic carbon (C), total nitrogen including organic and ammonium nitrogen (N) and total phosphorus (P) in the faeces were also analysed in this study. All the analysis was conducted as per the methodologies given in Republic of the Philippines (1980). For organic carbon determination, 0.2 gm of sample was treated with 1 normal potassium dichromate solution for 30 minutes. The excess potassium dichromate was titrated with standard ferrous sulphate solution and the organic carbon determined. Similarly, for total nitrogen determination, 0.2 gm of sample was digested with the mixture of potassium sulphate, copper sulphate, selenium and 30 ml concentrated H_2SO_4 in a Kjeldahl flask. After digestion, the mixture was treated with sodium hydroxide solution until it was strongly alkaline. It was then distilled into boric acid solution containing bromocresol green and methyl red. This mixture was titrated with standard sulphuric acid. Nitrates were neglected as they are often negligible compared to the total nitrogen. In the same way, for total phosphorus determination, 0.2 gm of sample was digested with concentrated sulphuric acid and potassium persulphate to destroy organic matter and was diluted to a fixed volume. An aliquot was taken and neutralized with NaOH. Total phosphorus was determined spectrophotometrically using ammonium molybdate and ascorbic acid.

Microbiological parameters

E. coli, total coliforms (TC), enterococci and *C. perfringens* as representatives of differently persistent microbes were

determined to infer potential bacterial pathogen die off conditions in faeces from 96 recovered samples. Three sub samples from each layer were mixed and homogenized manually using a sterile spoon. 10 gm of the homogenized sample was weighed into a calibrated 100 ml sterile bottle and filled to 100 ml with sterilized phosphate buffered saline solution (pH of 7.2). To make a suspension of the sample, the mixture was hand shaken and then stirred with a magnetic stirrer at high speed for 15 minutes. Samples were analysed within 6 hours of sampling.

E. coli and TC were cultured on Chromocult Coliform Agar (CCA, Merck Dermstadt, Germany), enterococci on m-Enterococci Agar (MEA, Difco, Detroit Michigan) and *C. perfringens* on Tryptose Sulphite Cycloserine (TSC, Merck) Agar including a supplement (4-methyl-umbelliferyl phosphate) for the detection of phosphatase activity and the colonies viewed under UV light. Media preparation was according to the manufacture's instruction. 1 ml of the faeces suspension was added to 9 ml sterile saline solution and appropriate dilutions were made. 0.1 ml of the respective diluted sample was spread homogeneously on the corresponding plates using a sterile glass rod. CCA and MEA plates were dry incubated at 37°C for 24 and 48 hours respectively. Enumeration of *E. coli*, TC, and *C. perfringens* colonies was performed as described in Byamukama *et al.* 2005. Due to limiting financial capacity, representatively grown colonies on selective cultivation media could not be selected for further detailed biochemical characterisation, thus enumeration of *E. coli*, TC, enterococci and *C. perfringens* refers to the presumptive level of identification.

Three fractions of *C. perfringens* i.e. vegetative cells and spores which germinate without heat activation (CPVS), germinating spores after heat activation at 60°C (CP60) and germinating spores after treatment at 85°C (CP 85) were analysed. The CPVS fraction was analysed on the prepared suspension samples. In addition, about 2 ml of the suspensions were pasteurised at 60°C and 85°C for 15 minutes in water baths to analyse for the CP60 and CP 85 fractions, respectively. TSC agar plates were put in an anaerobic jar and incubated at 44°C for 24 hours.

From the 96 recovered and pooled faecal samples 36 were randomly selected (i.e. 38% of the total sample number) for the determination of Helminthes using the Kato-Katz cellophane method (Katz *et al.* 1972).

Data analysis

CFU/wet gm of faecal material was calculated by use of the suspension factor (i.e. 1 gm in 10 ml), the pipetting factor (0.1 ml/plate) and the respective dilution steps. The weighted average of the respective colony counts was used when more dilutions were countable on the plates. The counts of the indicators were converted from CFU/wet gram of faeces to CFU/cm^3 to avoid any bias due to standardisation (Farnleitner *et al.* 2003). However, it was observed that the concentrations did not vary much when expressed per volume as the mass and the volume of the samples were generally about the same with the average conversion factor of 1.07. All counts were $\log_{10}$ transformed before further analysis. The reduction of the indicators over time was calculated with an assumption that the micro-organisms die-off can be estimated by a first order kinetics (Höglund 2001). SPSS 10.0 statistical package (SPSS Inc. Chicago, IL) was used for data analysis and the significance level at $p \leq 0.05$ was used.

RESULTS

Chemo-physical parameters

The chemo-physical parameters in the 4 investigated toilets are summarised in Table 1. There was no significant difference between the temperature in all the toilet units (median 24.0°C; range 19.5°C–32.8°C) and the ambient temperature (median 24.3; range 22.0°C–30.4°C). The pH in the various units ranged from 7.2 to 9.9. A significantly higher pH was recorded in toilet D with median 9.2 ($p = 0.001$, $n = 8 \times 12$) as compared to the other toilets (Table 1). Median moisture content of the toilets was in the range of 63.5% in toilet B to 70.4% in toilet A (Table 1), showing a significantly lower moisture content (median 63.5%, range 52.4%–75.0%) for toilet B ($p = 0.002$, $n = 4 \times 24$, Table 1). Generally, moisture increased with depth.

Microbial parameters

The concentrations of *E. coli* reflected the effect of the respective storage periods (Figure 1), but only 50% of the

Table 1 | Chemo-physical parameters of the different layers for each toilet unit. Presented are the median (*M*) and the range (*R*) values

		Temperature (°C)		pH		Moisture (%)		
Toilet Units	**Layers**	***M***	***R***	***M***	***R***	***M***	***R***	***n***
A	BT	23.4	20.0–24.4	8.8	7.6–9.3	73.8	67.7–78.7	8
	MD	24.6	22.1–26.6	8.5	8.3–9.1	72.4	63.1–76.7	8
	TP	24.6	21.7–26.8	9.2	7.7–9.3	68.0	62.3–71.4	8
B	BT	23.4	21.0–24.9	8.9	8.1–9.4	73.1	62.9–75.0	8
	MD	24.5	23.4 − 29.0	9.3	7.4–9.9	62.9	52.4–74.2	8
	TP	25.2	23.5–30.4	7.6	7.3–8.0	62.4	56.5–66.7	8
C	BT	23.2	19.5–23.7	8.4	7.7–8.9	71.2	62.0–75.4	8
	MD	23.5	23.2–32.8	8.6	7.2–8.9	69.3	65.6–71.6	8
	TP	23.1	22.7–27.5	8.9	7.4–9.3	70.2	67.7–73.4	8
D	BT	24.0	20.5–25.3	8.5	7.9–8.7	73.2	59.7–79.7	8
	MD	24.7	22.1–27.6	9.3	9.2–9.9	67.7	65.7–71.4	8
	TP	25.0	21.8–27.8	9.3	8.0–9.6	64.3	62.0–68.2	8
A	Pooled	24.3	20.0–26.8	8.8	7.6–9.3	70.4	62.3–78.7	24
B	Pooled	24.5	21.0–30.4	8.6	7.3–9.9	63.5	52.4–75.0	24
C	Pooled	23.3	19.5–23.2	8.6	7.2–9.3	70.1	62.0–75.4	24
D	Pooled	24.5	20.5–27.8	9.2	7.9–9.9	67.7	59.7–79.7	24

Layer BT = bottom layer, MD = Middle layer, TP = Top layer for each of the four test units; *n* = sample size.

investigated samples ($n = 96$) showed detectable concentrations. Except for the first 10 days, samples without detectable *E. coli* were observed during the whole investigation period. There was an increasing trend of not detectable (nd) *E. coli* in 0–20%, 50%, and 70–80% of samples for the first 3, 6 and 12 months, respectively. The lowest concentrations (nd–7.3×10^4 CFU/cm^3) were recorded in toilet A and the highest concentrations (nd–1.3×10^7 CFU/cm^3) in toilet C (Table 2). Linear regression analysis of the pooled data showed a significant reduction of *E. coli* ($p < 0.001$, $n = 48$) with an average $\log_{10}$ reduction of -2.4 with a 95% confidence interval ranging between -3.2 and -1.6 over the 6 months storage period (Table 3, Figure 1). For TC, only 15% of the total samples showed not detectable counts per cm^3. Regression analysis of the pooled data revealed a significant reduction of TC with time ($p < 0.001$, $n = 81$) (Table 3, Figure1).

The median counts of enterococci ranged from 2×10^5 CFU/cm^3 in toilet A to 2×10^6 CFU/cm^3 in toilet C (Table 2). The minimum concentrations ranged from 7.7×10^2 CFU/ cm^3 in toilet B to 6.9×10^3 CFU/ cm^3 in toilet C. None of the samples showed zero counts indicating the increased resistance of this indicator as compared to *E. coli* and TC. Linear regression analysis of the pooled data showed a significant reduction with time ($p < 0.001$, $n = 95$). The average $\log_{10}$ reduction was -1.0 with 95% confidence in the range between -1.5 and -0.57 over a 6 months storage period (Table 3, Figure 1).

C. perfringens was observed to be the most resistant bacterial indicator in this study. There were no significant differences between the determined different fractions of *C. perfringens*, i.e. CPVS, CP60 and CP85, (Table 2) however the CP 85 fractions showed a somewhat decreased trend (Figure 2). The median concentration for the vegetative cells was in the magnitude of 10^6 CFU/cm^3, except for unit A, which had a concentration in magnitude of 10^7 CFU/cm^3. For the CP85 fraction, the median concentrations ranged between the magnitude of 10^6 CFU/cm^3 in toilets A and D to the magnitude of 10^5 CFU/cm^3 in toilets B and C. *C. perfringens* did not show any significant reduction with varying storage periods.

An amount of 72% of the analysed samples ($n = 36$) showed presence of roundworm eggs. The median count was 264 eggs/cm^3 and ranged from 26–896 eggs/cm^3 of

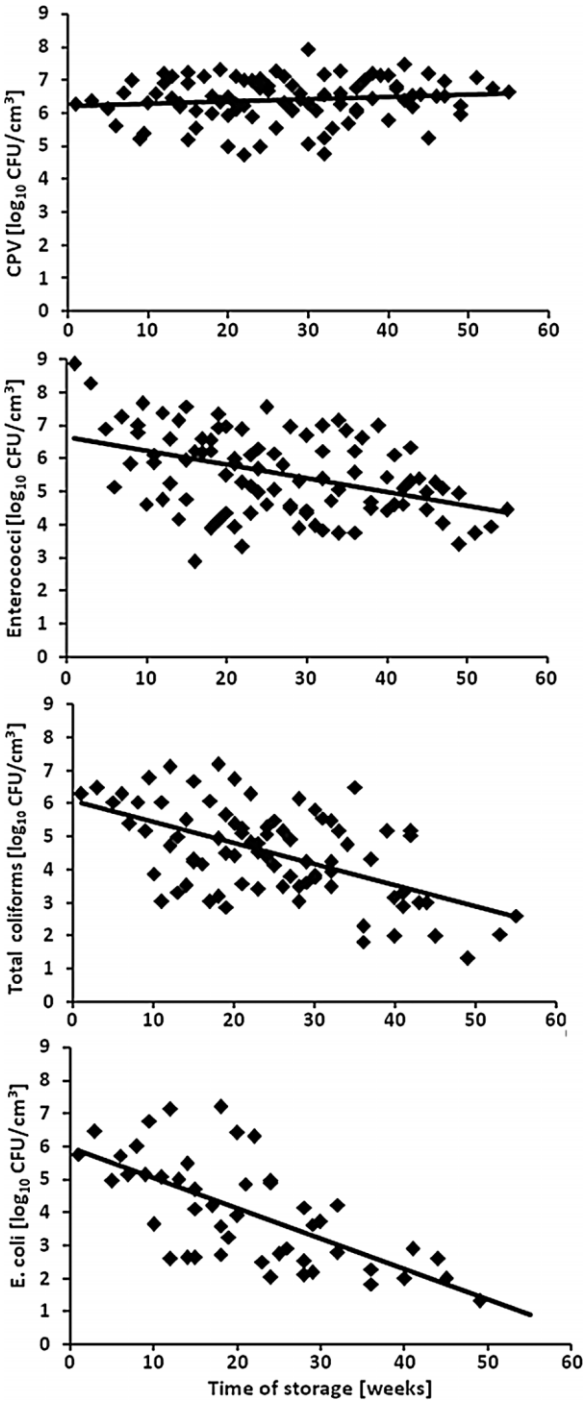

Figure 1 | Reduction of microbial indicators with time. Plotted are the $\log_{10}$ CFU concentration of each indicator with time in weeks. EC = *E. coli*; TC = total coliforms; Ent = enterococci; CPV = vegetative and germinating spores without heat activation of *C. perfringens*. Graphs for linear regression analysis is shown, for further statistical details refer to Table 2.

faeces. 19% of the samples also showed eggs of *Trichuris trichuria* with a median count of 78 eggs/cm^3 ranging from 26–144 eggs/cm^3. One sample showed tapeworm eggs and none showed hookworm eggs. No reduction of prevalence or abundance was observed with storage time (data not shown).

DISCUSSION

Chemo-physical parameters

Temperature is one of the major factors contributing to pathogen die off in faeces. Most enteric microorganisms die at temperatures >45°C and all types of pathogens except bacterial spores are killed at temperature of about 55°C–65°C (Höglund 2001). In a laboratory and pilot scale study, it was observed that faeces treated with ash or lime did not attain a high temperature (Vinneras *et al.* 2003). This could be a major reason for the low temperature determined during this study (median 24.0°C; range 19.5°C–32.8°C) as ash was used in all the four toilets (Table 1). We hypothesise that the addition of ash reduces the organic matter content in the faeces, thus reducing the rate of microbial degradation and heat production in the system. The observed high moisture level could also have contributed to low temperature due to evaporation and due to conduction of heat by the moisture. It should be noted that the measured temperatures in the faecal material of the studied toilets relate to the investigation period from May to August. Average temperatures in the vaults, spanning the whole seasonal cycle, are expected to be even lower for this hilly region.

The addition of ash, lime (Peasey 2000) or urea (Schönning 2004) elevates the pH in the stored faeces and according to Austin (2001), a pH value of 9 and above could be detrimental for all microbial growth. However, even though ash was used as the major additive in the studied toilets, pH was generally below 8 for three toilets and only one toilet (D) had a median value of >9. It could be possible that the amount of ash added was inadequate to create a homogenous and high pH range in the faecal heaps. Creating pockets of high and low pH might not be favourable for complete sanitization of the heap as pathogens could still persist in areas with lower pH.

Table 2 | Microbial levels of the indicators in the different toilets (CFU per cm^3 sample material). Presented are the median values (*M*) and ranges (*R*) of the pooled data for the four toilets ($n = 96$)

	Toilet Units									
	A		D		B		C			
Parameter	M	R	M	R	M	R	M	R		
E. coli	0	n.d.–7.3×10^4	n.d.	n.d.–1.4×10^5	5.0×10^1	n.d. $\times 10^0$–1.0×10^6	7.9×10^3	0.0×10^0–1.3×10^7		
TC	4.1×10^3	n.d. $\times 10^0$–5.0×10^6	2.8×10^3	n.d. $\times 10^0$–3.1×10^6	3.3×10^4	n.d. $\times 10^0$–2.0×10^6	2.7×10^4	0.0×10^0–1.3×10^7		
ENT	1.6×10^5	2.6×10^3–3.7×10^7	5.0×10^5	2.1×10^3–1.0×10^7	1.2×10^5	7.7×10^2–3.6×10^7	1.3×10^6	6.9×10^3–7.2×10^8		
CPVS	1.3×10^7	9.3×10^5–8.5×10^7	2.0×10^6	5.6×10^4–1.9×10^7	2.2×10^6	3.5×10^5–1.0×10^7	1.4×10^6	6.0×10^4–1.6×10^7		
CP60	1.0×10^7	3.1×10^4–7.6×10^7	5.2×10^6	9.4×10^3–5.0×10^7	2.1×10^6	4.8×10^4–1.9×10^7	5.5×10^5	4.6×10^4–7.5×10^6		
CP85	3.7×10^6	1.8×10^5–5.9×10^7	1.7×10^6	1.3×10^5–1.8×10^7	8.9×10^5	2.4×10^4–2.1×10^7	1.6×10^5	1.5×10^4–2.9×10^6		

TC = total coliforms; ENT = enterococci; CPVS = *Clostridium perfringens* vegetative cells and germinating spores without heat activation; CP60 = *Clostridium perfringens* after pasteurisation at 60°C; CP85 = *Clostridium perfringens* after pasteurisation at 85°C. n.d. = not detectable.

No significant effect by the chemo-physical parameters on the indicators was seen from the Spearman's correlation test (c.f. next paragraph, Table 4), most probably due to the high moisture content in this toilet as Peasey (2000) has reported that an increase in moisture >60% reduces the effect of high pH. However, it is important to investigate and further understand the effect of pH on the various survival stages of the microorganisms, as all might not be equally affected.

For a dehydrating system, a moisture content of <20% is desirable (Winblad 1997). Low moisture content or desiccation leads to microbial die off, since the microbes are unable to drive their metabolic processes in such an environment (Redlinger *et al.* 2001). However, distinction should be made between survival of resting stages and the active die off of the metabolising organisms. For micro-organisms capable of forming spores like *C. perfringens*, adverse environmental conditions might not lead to active die off as compared to less resistant vegetative forms. Common methods for reduction of moisture content in the dehydrating system is addition of soak materials like ash and saw dust and exposure to the sun for drying the faecal heap (Redlinger *et al.* 2001). In the toilets studied, ash was used for the dual purpose of increasing the pH as well as lowering the moisture. However, there was no pronounced reduction of moisture in any of the toilets (Table 1) indicating that the amount of ash used might not have been adequate. In some dehydrating systems, one side of the toilet is painted black to absorb more heat from the sun, to increase the temperature and to enhance moisture reduction. The investigated toilets did not have this provision and this could have affected the level of moisture reduction attained.

Reduction of indicator microorganisms

The major factors governing reduction of microorganisms in faeces, like temperature, pH and moisture, were apparently not in the optimal range and no single factor was found to have significantly contributed to the reduction of the indicators. There was no significant correlation between the bacteriological and the chemo-physical parameters detectable (c.f., Spearman's correlation analysis, Table 4). Past studies indicate that it is not any single factor

Table 3 | Linear regression analysis of $\log_{10}$ CFU indicator concentrations in toilets over storage time (weeks). Reduction coefficient (X) is given per week and calculated for 6 months

		$\log_{10}$ per week				$\log_{10}$ reduction (6 months)		
Indicator	***n***	***X***	***D***	**Sig. *X***	**Sig. *D***	**Average**	**95% confidence**	
EC	48	−0.0920	5.96	$P < 0.01$	$P < 0.01$	−2.39	−3.17	−1.61
TC	81	−0.0635	6.09	$P < 0.01$	$P < 0.01$	−1.65	−2.21	−1.09
ENT	95	−0.0399	6.61	$P < 0.01$	$P < 0.01$	−1.04	−1.51	−0.57

EC = *E. coli*; TC = Total Coliforms; ENT = Enterococcus, *X* = slope, *D* = deviation from 0 at time 0; Sig. *X* = significance of slope, Sig. *D* = significance of deviation, *n* = replicates.

which contributes to the reduction or inactivation of the tested microorganisms but rather continued exposure to a combination of all (Peasey 2000; Austin 2001). Studies conducted in Vietnam showed a complete destruction of *Salmonella typhimurium* 28B in 154 days and *Ascaris suum* eggs in 169 days with recorded moisture at 25%–60% and average temperature of 34°C (Scott 2002). It should be mentioned that there was a positive significant correlation between C and *E. coli*, TC and enterococci, respectively, observable (c.f. Table 4). Although partial re-growth activities cannot be excluded, the most likely explanation for this correlation can be given by the fact that pockets with increased organic carbon content could have contributed to lower die off rates due to more favourable survival conditions.

The relatively higher persistence of TC compared to *E. coli* can be deduced from the lower reduction rate. TC tend to remain viable with higher moisture and low pH and sun drying could be one of the options for further reduction of the TC due to elevated temperature and desiccation (Peasey 2000). However, it is important to note that the use of *E. coli* and TC as treatment indicators to test for the treatment efficiency is debatable due to their relatively short survival in the environment and inability to indicate for more persistent pathogens (Höglund 2001). In this study, these were used to predict the reduction conditions for the less resistant bacterial types such as for several important pathogenic groups found within the *Enterobactericeae*. It should be noted, that the high frequency of randomly occurring samples without detectable *E. coli*–even during the first phase of the storage periods -may be explained by a heterogeneous distribution of various micro-environments leading to contrasting die off conditions (i.e. environment composed out of alternating faecal and ash layers). In contrast, this differential effect was much weaker for TC, which may be explained by its higher resistance. For regression analysis samples without detectable concentrations of *E. coli* were not taken into account in order to achieve conservative die off estimates.

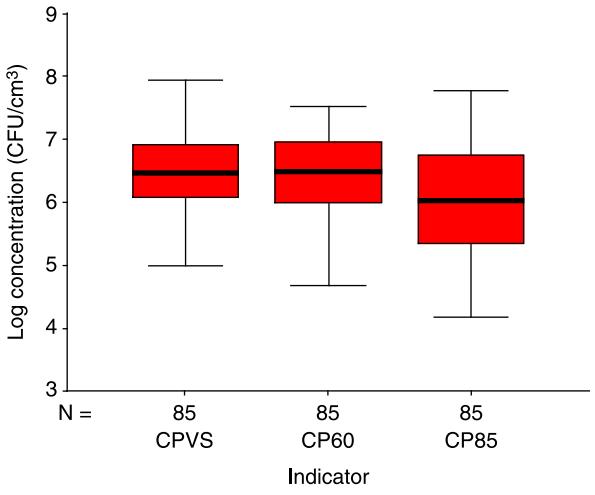

Figure 2 | Box plots of $\log_{10}$ CFU abundance of different fractions of *C. perfringens* revealed in the toilet samples. CPVS = vegetative and germinating spores without heat activation; CP60 = germinating spores after pasteurisation at 60, CP85 = germinating spores after pasteurisation at 85°C ($n = 85$).

Enterococci is considered to be a better indicator for more resistant faecal bacteria than the faecal coliforms due to its greater resistance. They have even been suggested as an indication of the presence of viruses in sludge (Höglund 2001). The observed lower die off rate (average $\log_{10}$ reduction = −1.0) of Enterococci in the samples over the storage period could therefore be an indication of potential survival of more resistant enteric pathogens. As these are resistant to high pH and desiccation, the storage conditions in these dehydrating systems were not appropriate for their sufficient reduction during the storage period. Moreover, in the studied toilets, the environment could have been more favourable to this microorganism where the moisture content was very high and the pH was generally below 9.

Table 4 | Spearman's (r_s) half matrix showing the correlation between various determined parameters ($n = 96$).

Parameters	Time	Temp	Moist	pH	C	N	P	EC	TC	ENT	CPVS	CP60
Time	1.00											
Temp	−0.09	1.00										
Moist	−0.37	0.37	1.00									
pH	−0.06	0.16	0.16	1.00								
C	−0.09	0.01	0.13	0.14	1.00							
N	−0.14	0.01	0.00	0.03	0.45	1.00						
P	−0.02	0.05	0.04	0.10	0.30	0.07	1.00					
EC	−0.55	0.00	0.10	0.16	0.32	0.14	0.01	1.00				
TC	−0.55	−0.01	0.06	0.17	0.43	0.29	0.17	0.58	1.00			
ENT	−0.39	−0.10	−0.22	−0.12	0.35	0.24	0.07	0.42	0.59	1.00		
CPVS	0.13	0.12	0.15	0.07	0.30	0.03	0.09	0.01	0.09	0.24	1.00	
CP60	0.23	0.11	0.03	0.02	0.11	0.08	0.05	0.11	−0.13	0.09	0.51	1.00
CP85	0.15	0.21	0.04	0.01	0.24	0.22	−0.01	0.20	−0.17	0.02	0.45	0.60

Temp = Temperature, Moist = Moisture, C = total organic carbon, N = total organic nitrogen, P = total phosphorous, EC = *E. coli*, TC = total coliforms, ENT = enterococci, CPVS = vegetative cells and germinating spores without heat activation of *C. perfringens*, CP60 = *C. perfringens* after pasteurisation at 60, CP85 = *C. perfringens* after pasteurisation at 85°C. Underlined coefficients are significant on the $p \leq 0.05$ level (Bonferroni correction was applied for multiple testing, $n = 13$).

C. perfringens spores are among the most resistant life stages of microorganisms and can remain viable in the environment for a very long time (Skanavis & Yanko 2001). No reduction was observed for the various *C. perfringens* fractions determined throughout the whole storage period (Figure 1), indicating that the conditions in the studied toilets were not sufficient to remove spores and other highly resistant live stages. However, a total destruction of *C. perfringens* is not essential for the use of faeces in the agricultural fields. The ineffectiveness of the storage conditions to reduce spore concentrations should only be taken as a strong indication of the potential survival of other very resistant survival stages formed by protozoan and parasitic pathogens. The high occurrence (72%) of *Ascaris*/helminthes eggs in randomly selected samples, and no reduction observed for *C. perfringens* calls for further investigation on the effective conditions for the removal of resistant forms during storage. Further studies on this topic should however also distinguish non-viable from viable parasitic forms, as only the last ones are of health relevance. No significant differences were observed between the various stages of *C. perfringens* (Figure 2). The basic intention of using differently treated fractions of *C. perfringens* was to distinguish between anaerobic vegetative cells from spores of different heat resistance as the combined use of these indicator fractions has previously been reported to be useful in the characterisation of faecal pollution of water resources (e.g. treatment history, age of faecal pollution; Bisson & Cabelli 1980). However, it should be noted that although pasteurisation removes vegetative forms, it can also break the dormancy–i.e. the so called heat activation–and allows more spores to germinate (Cato *et al.* 1986). The increased numbers of germinating spores at the CP 60 and CP 85 fractions obviously compensated for the removal of vegetative forms at the CPVS fraction. A combined application of *C. perfringens* fractions, as a result of different thermal treatment steps, thus cannot be recommended for further die off studies in bio-solids and the use of one single fraction (e.g. CP 60) appears to be sufficient.

Test for the distribution of the residuals from regression analysis did not deviate from the normal distribution and therefore first order decay was taken as a reasonable estimation for the indicators tested. It is possible that even though individual factors did not show significant effects on the indicators, there might have been a combined effect of all the factors together which led to the decrease of indicators integrated over time (Figure 1). Further analysis was therefore done to test the reduction against storage time of faeces in the different layers to evaluate for combined effects integrated over time.

For agricultural use, USEPA faecal coliform standard is $<1{,}000$ faecal coliforms/gm to meet the Class A biosolids (cited in Moe & Izurieta 2004). Following this guideline and to achieve this concentration in the present study, a 3 $\log_{10}$ reduction would be required (assumed initial concentration of about 6 $\log_{10}$ cfu/g) both for *E. coli* and TC. Assuming a first order decay and the calculated die off value, the estimated time of storage would be an average of 33 and 47 weeks with a confidence level of 95% ranging from a minimum of 25 and 35 to a maximum of 48 and 71 weeks for *E. coli* and TC, respectively. To achieve a 3 $\log_{10}$ reduction for enterococci, the estimated average time of storage would be 75 weeks with 95% confidence ranging from 52 to 136 weeks. According to recent WHO guidelines (2006) an even higher *E. coli* reduction target (6 $\log_{10}$) would be required for faecal excreta to achieve an equivalent health based target of $\leq 10^{-6}$ DALY (disability adjusted life years) per person and year as required for wastewater treatment. We did not attempt any extrapolation for the storage time required for an *E. coli* 6 $\log_{10}$ reduction target as the first order die-off kinetics is likely not applicable for this extended period of time. However, irrespective of the selected reduction target, the used storage time of 6 months was far too short for reaching an acceptable margin of safety in the prevailing storage conditions.

CONCLUSION

There was no observable correlation of temperature, pH and moisture with the reduction of the bacterial indicator microorganisms as these factors were not at the optimal level needed for an efficient performance of dehydrating toilets. This indicates a need for improvement in toilet design and in use of the toilets to improve the storage conditions of the faeces in order to increase the reduction of microorganisms. The estimated pathogen die off conditions in the investigated toilets, as derived by the faecal indicators *E. coli*, TC and enterococci, cannot be considered sufficient for safe handling and use of the biosolids when a 6 month storage period is applied in the prevailing storage conditions. The conclusions recovered are strongly supported by the new guidelines for the safe use of excreta (WHO 2006) recommending a >6 months storage period only if alkaline treatment conditions (pH >9) under appropriate temperature (>35°C) and moisture conditions ($<25\%$) apply. In case of ambient storage conditions ranging between 2°C and 20°C, a storage period of 1.5 to 2 years is suggested (WHO 2006). Recommended WHO storage conditions are in general agreement with recent results from quantitative microbial risk assessment, using various bacterial, viral and parasitic indicator pathogens and different exposition scenarios, suggesting that infection risks can become unacceptably high if storage periods and/or conditions are insufficient (Schönning *et al.* 2007). The high detection rates and numbers of helminthes eggs, found in the investigated faecal samples, furthermore points to an increased potential of faecal transmittable parasitic diseases in this area. Thus, further work in the studied region has to evaluate the most beneficial improvement measures in toilet design, operation, storage conditions/time, and possible post treatment activities in order to assure a safe handling and reuse of excreta. The generally achievable reduction efficiency in dehydrating toilets however should not be underestimated. With improved storage conditions a better reduction of enteric microorganisms is likely achievable as it has been reviewed previously (WHO 2006). For example in a Chinese study a >7 $\log_{10}$ reduction for bacteriophages and faecal coliforms were recorded after six months, even though the temperature was very low (-10°C to 10°C), resulting in partial freezing of the material (Wang 1999).

ACKNOWLEDGEMENTS

This study was funded by the Austrian Development Cooperation (ADC) within the Master of Science Programme "Limnology & Wetland Ecosystems" at the Institute for Limnology, Austrian Academy of Sciences (ÖAW) in co-operation with UNESCO-IHE, Institute for Water Education Delft, the Netherlands (EZA-Project 612-00/04) and supported by the Austrian Science Fund within the translation research project L414-B03 granted to AHF.

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First received 24 December 2007; accepted in revised form 28 February 2008. Available online October 2008.