



Microbial risk from source-separated urine used as liquid fertilizer in sub-tropical Australia

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ABSTRACT

The inactivation rates of *Escherichia coli* (*E. coli*) and MS2 phage were determined in fresh undiluted, diluted and six months aged stored urine samples at three temperatures ranging from 15 to 35 °C in a subtropical region of Australia. In addition, Quantitative Microbial Risk Assessment (QMRA) calculations were undertaken to estimate the storage times that would be required to reduce the risk of infection by *Campylobacter jejuni* (using *E. coli* inactivation data) and rotavirus (using MS2 phage data) to an annual probability of infection of 10^{-4} during irrigation and consumption of lettuce. Higher inactivation rates were observed at a higher temperature (35 °C) compared to lower temperatures (15 and 25 °C) for both *E. coli* and MS2 phage. Stored urine sample also showed higher rates of inactivation for both *E. coli* and MS2 compared to undiluted and diluted urine samples at all temperatures. QMRA calculations indicated that inactivation of both bacteria and viruses to meet the health target of an annual probability of infection of 10^{-4} would take approximately four months at 15 °C, 10 days at 25 °C and five days of storage times at 35 °C. The results also indicated that an increase in temperature has a more substantial effect on reducing storage time than varying the urine dilution for both *E. coli* and MS2 phage. Combining the QMRA-based approach with pathogen reduction interventions as presented in this study provides a range of management options for regulators, and may reduce barriers to the application of source-separated urine associated with long storage times.

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1. Introduction

The reuse of human urine as fertiliser has received considerable attention due to the following primary benefits: (i) it contains 80–90% of the nitrogen, 50–65% of the phosphorus, and 50–85% of the potassium (Heinonen-Tanski and van Wijk-Sijbesma, 2005; Johansson et al., 2002); (ii) it can reduce water use; (iii) it reduces nutrient loads on wastewater treatment systems, thereby reducing the cost and energy for treatment; and (iv) it contributes to closing the energy intensive nutrient loop for manufactured (nitrogen) and mined (phosphorous and potassium) nutrients. The separation of urine from wastewater is possible with special toilets developed in Europe commonly referred to as urine-separating toilets (USTs) or source-separation toilets (Lienert and Larsen, 2010). These toilets are comprised of two chambers; the first chamber collects the urine while the second chamber collects the feces. Consequently, urine and flush water (approximately 2 L per flush) is collected in a storage tank, and after appropriate treatment, is transported to designated agricultural areas for application as fertilizer to grow crops.

Substantial concentrations of *Escherichia coli* (Andreu, 2005), *Salmonella typhi* (Feacham et al., 1983), *Salmonella paratyphi* (Feacham et al., 1983), *Schistosoma haematobium* (Feacham et al., 1983), *Mycobacterium tuberculosis* (Grange and Yates, 1992), and polyomaviruses (Bofill-Mas et al., 2000) could be excreted by an infected individual. Unfortunately, little is known regarding the environmental transmission of these urinary excreted pathogens. In addition, feces collected in the rear chamber can easily cross-contaminate the urine collecting section of the toilet, and hence, the urine in the storage tank (Schönning et al., 2002). High concentrations of bacterial, viral, protozoa, and helminths can be excreted in human feces (Fane, 2004; Prüss et al., 2002). A recent study reported the presence of multiple bacterial (*Aeromonas* spp., *Shigella* spp., *E. coli* O157:H7), and viral pathogens (polyomavirus, rotavirus, and adenovirus) in urine storage tanks in South Africa (Bischel et al., 2015). Once in the external environment, these pathogens, especially pathogenic bacteria, generally inactivate rapidly due to environmental stresses. However, certain viruses, protozoa, and helminth eggs can survive for several months with the potential to cause infections in humans. Handling of urine with high concentrations of pathogens may pose health risks to collection personnel, farmers, local communities and product consumers.

Extended storage of urine in closed storage tanks has been recommended as a safety measure to inactivate pathogens found in source-

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separated urine (Maurer et al., 2006). The inactivation of fecal indicators and pathogens in urine during storage has been evaluated at temperatures of 4 and 20 °C in Sweden (Höglund, 2001). Based on the study results, it has been recommended that if urine is used as fertilizer for commercial farming, higher infection risks may be involved, and, therefore, urine has to be stored at least for six months. In colder climates, a longer storage time may be required (WHO, 2006). The factors that may affect the inactivation of pathogens in the stored urine are primarily temperature, dilution, elevated pH, and free ammonia (Chandran et al., 2009; Dercey, 2015; Höglund, 2001; Nordin et al., 2013; Vinnerås et al., 2008). USTs have been used in Europe for years (Lienert and Larsen, 2010), but are yet to be accepted as a viable technology in Australia, partly because of the regulatory concern with public health risks. An understanding of the persistence of enteric pathogens in stored urine as a function of time is essential to minimize public health risks associated with the handling and reuse of source-separated urine.

Quantitative microbial risk assessment (QMRA) is a process of estimating the human health risk associated with defined scenarios involving exposure to specified pathogens (Haas et al., 2014). It involves characterization of microbial pathogens in water matrices followed by an assessment of exposure to these pathogens. The probability of infection (P_{inf}) is then estimated for an exposed population based on a dose-response model (if available). Risks are finally characterized based on the frequency of the exposure events to estimate a total risk per year, and evaluated against the health target set by the regulator. In some cases, Disability-Adjusted Life Years (DALYs) are estimated from the risk of infection by including the likelihood of illness, and the impact on quality of life from its severity (Havelaar et al., 2000).

The primary aim of this research study was to evaluate the inactivation rates of a bacterial indicator (*E. coli*) and a viral surrogate (MS2 phage) in fresh urine collected from healthy humans, and stored urine from an eco-village at different temperatures and dilutions. Furthermore, QMRA calculations were undertaken to determine the health risk implications in terms of the storage times that would be required to reduce the risks to a target annual probability of infection of 10^{-4} as recommended by the US EPA (Regli et al., 1991). Finally, several possible management intervention measures were discussed to achieve the health risk target.

2. Materials and methods

2.1. Sources of urine samples

Fresh human urine samples were collected from four healthy adults (two males and two females). Six months aged stored urine samples were collected from a centralized 20 kL polyethylene storage tank at the Currumbin eco-village, Gold Coast, Southeast Queensland, Australia. The Eco-village is known for its sustainable residential development and is often viewed as an exemplar for future urban development (Hood et al., 2009). A total of 12 USTs were installed at the eco-village. The toilets were Gustavsberg Nordic 393-U units that were selected based on several criteria to maximize the benefit of urine separation. These include a low flush volume mixing with the urine, lack of specialized moving parts, and compatibility with Australian plumbing fittings. The toilets are connected to 300 L polyurethane storage bladders via 50 mm polyethylene pipes. The volume of flush water entering the storage bladder was calculated to be 200 mL for a half cistern flush of 2 L, and 400 mL for a full flush of 4 L (Hood et al., 2009). For a 300–500 L storage bladder, the fill time is approximately one month for an average household size of

three people urinating 1.5 L per day (combined with 2.5 L flush water per person per day) (Beal et al., 2008). After one month, urine deposited in the storage bladder is transferred to a centralized storage tank (Beal et al., 2008). The remaining toilet waste discharges into a cluster scale wastewater treatment plant which produces A⁺ standard wastewater (highest quality of recycled water used for non-drinking purposes). This wastewater is used for toilet flushing and other non-potable uses (The State of Queensland Environmental Protection Agency, 2005). The collected fresh human and six months aged stored urine samples were transported to the laboratory on ice for the inactivation experiments.

2.2. Experimental set up

Inactivation experiments were conducted with *E. coli* (ATCC 9637) and MS2 phage (ATCC 15597-B1) with *E. coli* Famp (ATCC 700891) host. The inactivation of *E. coli* and MS2 phage was assessed in: (i) fresh undiluted urine; (ii) fresh urine diluted with deionised water at a ratio of 1:1; (iii) fresh urine diluted with deionised water at a ratio of 1:3; and (iv) six months aged stored urine with toilet flush water. A single pure *E. coli* colony was grown overnight at 37 °C in Nutrient Broth (Oxoid, UK) in a shaking incubator at 100 rpm. The cells were centrifuged at 4500 g for 5 min and washed twice in 20 mL sterile phosphate buffer saline (PBS). The concentrations of *E. coli* in the suspension were determined by a spread plate method (Ahmed et al., 2014a).

MS2 phage (ATCC 15597-B1) was recovered by adding ATCC recommended broth (Tryptone yeast extract glucose broth 271) to a freeze-dried phage vial. MS2 was grown by preparing a series of 10-fold dilutions of the freeze-dried stock, and plating them with *E. coli* Famp host using the Single Agar Layer US EPA Method 1602 (US EPA, 2001). After 24 h of incubation, 10 mL of deionised water was added to the top of the plates and mixed gently. The phage biomass was collected using a sterile pipette and transferred to a sterile centrifuge tube. The biomass was then filtered through a 0.22 µm Milipore filter, and stored at –80 °C.

Centrifuge tubes were filled with 50 mL undiluted, diluted (1:1, 1:3), and stored urine samples. *E. coli* and phage biomass were added to each tube to a final concentration of 1.7×10^7 CFU per mL, and 8.0×10^6 PFU per mL. After seeding, the tubes were rapidly mixed, sealed, and incubated at 15 °C ($\pm 1^\circ$), 25 °C ($\pm 1^\circ$), and 35 °C ($\pm 1^\circ$) in the laboratory. To avoid interference between *E. coli* and MS2 phage, two sets of experiments were undertaken. In each experiment, all the samples were tested in triplicate. A set of unseeded urine samples (undiluted, diluted 1:1, diluted 1:3 and stored urine) was incubated at 15, 25, and 35 °C to measure the pH level.

2.3. Microbiological analysis

The surviving concentrations of *E. coli* and MS2 phage were determined after 0, 1, 3, 7, 15, 30, 50, 70 and 100 days for all seeded urine samples that were incubated at the three different temperatures. In brief, 1 mL of seeded urine sample was taken from each 50 mL tube and serially diluted with PBS. The concentration of *E. coli* was enumerated by a spread plate method. Sample serial dilutions were placed on modified mTEC agar plates (Difco, Detroit, MI). Agar plates were incubated at 35 °C for 2 h to recover stressed cells, followed by incubation at 44 °C for 22 h (US EPA, 2002). The concentration of MS2 in each tube was enumerated by plating at least four different dilutions in triplicate per US EPA Method 1602 (US EPA, 2001). No further analyses were undertaken if two subsequent sampling occasions gave zero CFU or PFU per mL of sample.

2.4. Data analysis

The inactivation rate constant K was determined by fitting the exponential inactivation function (Eq. (1)).

$$C_t = C_0 \times 10^{-Kt} \quad (1)$$

where C_0 is the initial *E. coli* and MS2 concentration at time = 0, C_t is the *E. coli* and MS2 concentration at time = t .

A “best fit” curve-fitting approach was used where, for each indicator organism $\times$ treatment, all concentrations determined for each replicate at each sampling occasion were transformed to $\log_{10}$ values, and plotted against time. The function $\log C_t = \log C_0 - Kt$ was fitted by linear regression using Table Curve 2D software (Jandel Scientific, San Rafael, California, USA).

In some experiments, concentrations of *E. coli* at several sampling occasions exceeded the seeded concentrations, indicating regrowth of *E. coli* in the experimental tubes. This complicated the fitting of the regression line to the *E. coli* data. Hence, this initial growth phase was excluded from the regression analysis. Only data points above the assay limit of detection (1 CFU or PFU per mL) were used in calculating the inactivation rate constant K . An analysis of variance (ANOVA) was performed on the K inactivation rate of *E. coli* and MS2 in undiluted, diluted and stored urine samples incubated at for various temperatures. The critical P -value for the test was set to 0.05.

2.5. Health risk assessment

A Quantitative Microbial Risk Assessment (QMRA) was conducted to understand the health risk due to the presence of pathogens in stored urine used as a liquid fertilizer, as per recommended guidelines (Tilley et al., 2014; Joensson et al., 2004; WHO, 2006). The risk assessment was conducted with the goal of identifying target storage times to reduce risks to an appropriate level. The QMRA followed a four-step procedure as described elsewhere (Haas et al., 1999) and is discussed below.

2.5.1. Step 1 – hazard Identification

Source separation of human urine occurs in toilets with two bowls, one in the front of the bowl for urine collection and one at the rear of the bowl for the collection of fecal material (Jönsson et al., 1997). Health risk from pathogens transmitted in the urine itself is generally considered low (Schönning et al., 2002). However, the type and concentrations of pathogens in feces are unknown. Therefore, it is impractical to set human health-based targets for all pathogens that may be present in the feces. Instead reference pathogens such as *Campylobacter jejuni* (for bacteria) and rotavirus (for viruses) were chosen as representative. *C. jejuni* is considered to be the most common cause of bacterial gastroenteritis in Australia (OzFoodNet Working Group, 2003), while rotavirus poses a major threat of viral gastroenteritis worldwide (NRMMC-EPH-AHMC, 2006).

2.5.2. Step 2 – dose response

For bacteria and viruses, the Beta-Poisson relationship was chosen (Haas et al., 1999), where probability of infection (number of infections per person) (P_{inf}) for pathogen i (for i where $cj = C. jejuni$ and $rv =$ rotavirus) is calculated from:

$$P_{inf} = 1 - \left(1 + \frac{D}{\beta_i}\right)^{-\alpha_i} \quad (2)$$

where α_i and β_i are dose response parameters for the respective pathogens and D is the mean dose or number of pathogens ingested.

2.5.3. Step 3 – exposure assessment

The UST system is a closed system, so there is minimal risk of uncontrolled leakage of urine into the environment being a source of exposure. Two ingestion routes were considered as likely for UST households in Queensland. These scenarios were accidental ingestion during handling of the urine when irrigating gardens and ingestion during consumption of uncooked lettuce irrigated with urine from the UST. Lettuce was considered as an index crop because of its high water retention compared to other crops due to its large and uneven surface area, its high probability of being eaten raw, and short shelf life that bounds the possible time between harvest and consumption. These factors minimize pathogen decay compared to other produce, making it a conservative choice for QMRA so that predicted storage times can be reasonably assumed to be sufficiently public health-protective for other crop choices. Some other vegetables can have marginally higher water retention volumes, however, the average Australian daily serving size for lettuce is more than double that of other produce such as broccoli, cabbage, and cauliflower, which influences produce risk assessment to a greater extent than the volume retained (Barker et al., 2013). The dose of pathogens ingested will depend on the concentration of pathogens in the urine at the time of contact, decay of pathogens, and the volume of urine ingested. The model for daily pathogen dose in source-separated urine is described in Eqs. (3) and (4). The daily dose of pathogens therefore represents the number of pathogens accidentally consumed either by a person irrigating produce on an irrigation day (Eq. (3)), or the number of pathogens consumed by a person consuming a single serving of lettuce in one day (Eq. (4)). It is noted that the daily dose of pathogens results from the use of urine from a centralized storage tank.

$$D_{AI} = \frac{R_i}{365 \text{ days}} C_{f,i} E_i C_u 10^{-K_u t_u} V_{AI} \quad (3)$$

Where D = daily dose of pathogen i ($i = cj$ for *C. jejuni* or rv for rotavirus) from accidental ingestion of source-separated urine during irrigation (number of pathogens per day), R = annual disease incidence rate (%), C_f = pathogen concentration in the feces, E = excretion time (days), C_u = Concentration of feces in urine (mg feces per L urine), K_u = decay constant for pathogen in urine (per day), t_u = storage time in container (days), and V_{AI} = accidental ingestion volume (L urine). The models were simulated for various hypothetical storage time (t_u) to determine a sufficient time for public-health protection.

$$D_{CL} = \frac{R_i}{365 \text{ days}} C_{f,i} E_i C_u 10^{-K_u t_u} 10^{-k_{f,i} t_f} V_R I \quad (4)$$

For the lettuce exposure scenario, V_R = volume retained per gram of lettuce during irrigation (L per gram), I = gram consumed per person per day (grams per day), $k_{f,i}$ = in-field decay constant (per day), t_f = withholding time between harvest and consumption (days). Eqs. (3) and (4) were substituted into the dose response model (Eq. (2)) to

obtain a daily P_{inf} . Parameters are described below and summarized in Table 1.

2.5.3.1. Estimating concentrations of pathogens in the urine

Fecal contamination of urine in source-separated toilets cannot be modeled in the same way as traditional toilets due to the fact that the liquid and solid wastes are separated during use. However, fecal indicator bacteria are not considered to be reliable for estimating the quantity of fecal cross-contamination in source-separated urine (Höglund et al., 1998). Alternatively, fecal sterols have been demonstrated as appropriate indicators of fecal contamination in source separated urine (Schönning et al., 2002). In a study of 36 source separated urine samples from eco-villages, workplaces, schools, and households in Sweden, 10 samples (eight confirmed contaminated samples and two indeterminate samples later classified as contaminated) were determined to be contaminated with human feces using fecal sterol profiles (Schönning et al., 2002). Individual values were provided for the concentrations of coprostanol, which were converted to concentrations of feces in urine using a conversion factor of 4 μg coprostanol per mg feces. In this way, coprostanol concentrations were converted to the mass of feces per unit urine in centralized storage tanks (C_u). Pathogens were assumed to be homogeneously distributed in contaminated feces. Schönning et al., 2002 noted that the sludge volume in relation to the liquid portion in centralized tanks was small and thus will only slightly increase the risk if mixed into the liquid, which is unlikely to occur. For the purposes of this work, we assume the liquid in centralized storage tanks is well-mixed and that pathogens are homogeneously dispersed in the liquid as a result of fecal mixing with urine. Using the total number of samples ($n = 36$), including positive and negative samples, an interval-censored lognormal distribution was fit to the data using the method detection limit of 5 μg coprostanol per L (1.25 mg feces per L urine). This distribution is assumed to be representative of the concentration of feces in urine in a central storage tank at time $t_u = 0$ days, accounting for the fact that feces become mixed with and diluted by the urine and are not present in all samples taken from centralized storage tanks. Distribution fitting was performed using the fitdistrplus package in R (www.rproject.org). The log-normal distribution is a commonly used distributional form for environmental data fitting for concentrations of pathogens in water (US EPA, 1991).

The distributions for concentrations of *C. jejuni* and rotavirus in feces were derived from a previous QMRA (Höglund et al., 2002b). It is noted that the microbiological units were not specified in the cited references used to derive the distributions (Table 1) and were reported in number of pathogens per g feces with the exception of Ward et al., (1986) that reports units in focus forming units (FFU). Therefore, it was necessary to assume that the number of pathogens per gram of feces in the cited references was equivalent to PFU and CFU units used for enumeration in the current study. The degree to which pathogens were present in feces was estimated using epidemiologic modeling as indicated in Eq. (3) (Gerba, 2000; Ottoson and Stenstrom, 2003; Petterson et al., 2016). The annual incidence of infection and duration of pathogen excretion were used to correct the overall fecal shedding rate.

Pathogen inactivation rates – The *E. coli* and MS2 phage inactivation data from this study was used to indicate the inactivation rate of *C. jejuni* and rotavirus, respectively, in undiluted and diluted urine samples stored at different temperatures. For the QMRA, point estimates for the inactivation rates were used. It was assumed that the log-linear inactivation rates exhibited the same behavior for the entire duration of the simulated storage time (i.e. continued to behave linearly over the duration of urine storage).

Table 1

Monte Carlo simulation parameters used in the calculation of QMRA for source-separated urine.

Description	Symbol	Units	Parameters	Distribution	References
Yearly incidence rate, <i>C. jejuni</i>	R_{cj}	%	2.4	Point estimate	Petterson et al. (2016), Hellard et al. (2000)
Yearly incidence rate, rotavirus	R_{rv}	%	1.28	Point estimate	Petterson et al. (2016), Hellard et al. (2000)
<i>C. jejuni</i> concentrations in feces	$C_{f,cj}$	Number per gram feces	$\mu = 16.11$, $\sigma = 2.15$	Lognormal	Feachem et al., (1983), Höglund et al. (2002b)
Rotavirus concentration in feces	$C_{f,rv}$	Number per gram feces	$\mu = 23.02$, $\sigma = 2.15$	Lognormal	Bishop, (1996), Gerba et al. (1996a), Ward et al. (1986), Höglund et al. (2002b)
Excretion duration <i>C. jejuni</i>	E_{cj}	days	$\mu = 2.295$, $\sigma = 0.909$	Lognormal	Feachem et al., (1983)
Excretion duration rotavirus	E_{rv}	days	$\mu = 1.956$, $\sigma = 0.833$	Lognormal	Feachem et al., (1983), Gerba et al. (1996a))
Fecal contamination	C_u	mg feces per L urine	$\mu = -1.106$, $\sigma = 2.244$	Lognormal ^a	Schönning et al. (2002)
Inactivation K (Log ₁₀ -linear) in stored urine	k_u	Per day	K values in Table 2	Point estimate	This study
In-field <i>C. jejuni</i> decay (Log ₁₀ -linear)	$k_{f,cj}$	Per day	$\mu = 1.09$, $\sigma = 0.46$	Normal, truncated at 0	Stine et al., (2005)
In-field rotavirus decay (Log ₁₀ -linear)	$k_{f,rv}$	Per day	$\mu = 0.47$, $\sigma = 0.037^b$	Normal, truncated at 0	Petterson et al. (2001), Patterson et al. (2002)
Time in field after irrigation before harvest	t_f	Days	2	Point estimate	Barker et al. (2013)
Accidental ingestion volume	V_{AI}	mL	1	Point estimate	Asano et al. (1992), Höglund et al. (2002b)
Volume retained by lettuce	V_R	mL per gram	$\mu = 0.108$, $\sigma = 0.019$	Normal, truncated at 0	Shuval et al. (1997)
Accidental ingestion events per year	n_{AI}	Events per year	90	Point estimate	NRMMC-EPHC-AHMC (2006, Table 3.3)
Consumption of lettuce per day	I	Gram per day	40	Point estimate	NRMMC-EPHC-AHMC (2006, Table 3.3)
Lettuce consumption events per year	n_{CL}	Days per year	70	Point estimate	NRMMC-EPHC-AHMC (2006, Table 3.3)
<i>C. jejuni</i> Beta-Poisson model for infection	α_{cj}	Unitless	$\alpha = 0.145$, $\beta = 7.58$	Point estimates	Haas et al. (1999)
Rotavirus Beta-Poisson model for infection	β_{cj} α_{rv} β_{rv}	Unitless	$\alpha = 0.253$, $\beta = 0.426$	Point estimates	Haas et al. (1999)

^a Lognormal parameters mean, standard deviation (μ , δ) calculated from population (normal) parameters ($\bar{x}$, s) using standard formulae as follows: $\mu = \ln(\bar{x}^2/(s^2 + \bar{x}^2)^{1/2})$, $\delta = [\ln(1 + (s^2/\bar{x}^2))]^{1/2}$, where $\bar{x}$ is the sample mean and s^2 is the sample standard deviation.

^b Note that Petterson et al. (2002) discusses differences between Log₁₀ and lognormal (base e) fits to the data. The Log₁₀ form was used for consistency with the other K

values reported here using the functional form for decay corresponding to the Log_{10} parameters ($C = C_0 10^{-kt}$). A comparison of simulated risks using lognormal parameters reported in Petterson et al. (2002) ($\mu = 1.07$, $\sigma = 0.07$) with the lognormal form ($C = C_0 e^{-kt}$) produced identical results.

2.5.3.2. Estimating the volume of urine likely to be ingested on contact

Consumption of lettuce. Urine was assumed to be applied to lettuce that would be consumed raw after a two-day withholding period as the plants are not likely to thrive after a two-day period without irrigation during warm weather (Barker et al., 2013). In-field decay constants were assumed, however, post-harvest removal was not considered as the principal management option explored here is the storage time. A mean retention volume of 10.8 mL per 100 g lettuce following immersion was chosen for urine application (Shuval et al., 1997). The suggested daily serving size per person in the Australian Guidelines for Water Recycling is 40 g and leafy vegetables are estimated to be eaten 70 times per year (NRMCC-EPH-AHMC, 2006).

Accidental ingestion during application of urine. The volume ingested during irrigation activities was assumed to be of 1 mL (Höglund et al., 2002b) and to occur 90 times per year (NRMCC-EPH-AHMC, 2006).

2.5.4. Step 4 – risk characterization

The mean dose (D) was used to calculate the risk of infection per person per day using the dose-response relationship for the specific pathogen (Eq. (2)). The annual risk (Eq. (5)) was then computed for each simulated urine storage time (t_u). To simulate annual risks, the product of n randomly sampled daily risks with replacement were computed as per the preferred method for an annualizing probability of infection (Karavarsamis and Hamilton, 2016) using 50,000 iterations. All computations were performed in R and using the mc2d package (Pouillot et al., 2010).

$$1 - \prod_{i=1}^{n,i} (1 - P_{\text{daily}}) \quad (5)$$

3. Results

3.1. pH of the undiluted, diluted and stored urine samples

The initial pH (mean $\pm$ SD) of the undiluted urine, diluted 1:1, diluted 1:3 and stored urine samples were 6.8 ± 0.11 , 6.7 ± 0.12 , 6.8 ± 0.17 and 9.0 ± 0.14 , respectively. The pH level was measured weekly from the unseeded control sample for each temperature. The pH of the fresh urine, diluted 1:1, diluted 1:3 increased to 9.1 ± 0.14 , 8.9 ± 0.11 and 8.7 ± 0.13 , respectively, after nine weeks of storage. The pH of the stored urine remained same throughout the inactivation experiment.

3.2. Inactivation rates

Mean concentrations (CFU or PFU per mL) of *E. coli* and MS2 phage as a function of time (days) in undiluted, diluted (1:1 and 1:3) and stored urine samples at different temperatures are shown in Figs. 1 and 2, respectively. The exponential relationship for determining the K inactivation rates fitted the data showing R^2 values ranging from 0.68 to 0.98 (Table 2), once the initial growth phase observed for *E. coli* was excluded (Fig. 1). This growth phase accounted for a maximum of 10 days at 15 °C, 5 days at 25 °C and 1 day at 35 °C.

Higher mean inactivation rates were observed for higher temperatures. *E. coli* rapidly inactivated at 25 and 35 °C for the stored urine ($K = 2.37$ per day at 25 °C and $K = 6.15$ per day at 25 °C), undiluted ($K = 0.31$ at 25 °C and $K = 3.04$ at 35 °C), and diluted urine ($K = 0.31$ to 0.32 at 25 °C and $K = 1.75$ to 3.83 at 35 °C). A similar trend was also observed for MS2 which inactivated rapidly at 25 and 35 °C (Table 2). MS2 inactivated more gradually by increased temperature than *E. coli*. This is demonstrated by the higher inactivation rates of MS2 compared to *E. coli* at 25 °C.

Stored urine also showed higher mean inactivation rates for *E. coli* ($K = 0.89$ to 6.15 per day) compared to undiluted ($K = 0.20$ to 3.83 per day), and diluted 1:1 and 1:3 ($K = 0.21$ to 3.83 per day) at 15, 25 and 35 °C. MS2 phage in stored urine samples also showed higher mean inactivation rates ($K = 1.88$ to 2.15 per day) compared to undiluted urine ($K = 0.38$ to 1.21 per day), and diluted 1:1 and 1:3 ($K = 0.07$ to 1.25 per day) at 15 and 25 °C. However, *E. coli* 1:3 diluted urine showed a lower inactivation rate ($K = 1.75$ per day) compared to undiluted ($K = 3.04$ per day), and diluted 1:1 ($K = 3.83$ per day) urine at 35 °C. For MS2 phage, K values decreased across treatments at 15 °C, with K for stored > undiluted > diluted 1:1 > diluted 1:3. MS2 inactivation rates for undiluted, diluted 1:1 and diluted 1:3 urine samples were similar and lower than stored urine at 25 °C. The MS2 phage inactivated more rapidly at a higher temperature (35 °C) with all dilution treatments showing similar mean inactivation rates ranging from 3.10 to 3.14 per day compared to 15 and 25 °C ($P < 0.05$). This trend was also observed for *E. coli* (for undiluted and diluted urine samples) at 25 °C but not at 35 °C. The inactivation K for undiluted and diluted urine samples at 15 and 25 °C significantly varied from 35 °C ($P < 0.05$).

3.3. Quantitative microbial risk assessment

A scenario analysis was undertaken to examine the effect of temperature and dilution rate on the annual P_{inf} for *C. jejuni* and rotavirus during accidental ingestion, and consumption of urine-irrigated lettuce. Urine storage time is the principle management intervention considered, and as a result, the simulations were performed over a range of hypothetical simulated storage times (t_u) to determine variability in annual risks. Annual risk (and therefore storage time) variability cannot be determined adequately if storage time is solved for directly using a single risk benchmark value. The effect of increasing storage time on the annual P_{inf} is shown in Figs. 3 and 4. For *C. jejuni*, a storage time of 25 days was sufficient to reduce the 95th percentile for an annual probability of infection due to accidental ingestion during irrigation with stored urine to less than 10^{-4} at all temperatures (Fig. 3a–c). A storage time of 20 days was sufficient to reduce the 95th percentile to less than 10^{-4} probability of infection for consumption of lettuce irrigated with stored urine at all temperatures (Fig. 3d–f). Annual risks for *C. jejuni* decreased with increasing temperature. The difference in risk reduction was greater when increasing the temperature from 25 to 35 °C compared to 15 to 25 °C, indicating that the relationship between temperature and inactivation K may not be linear. Dilution did not have a substantial impact on the annual risks for *C. jejuni*, which is reflected in the relatively similar values for K derived from those experiments.

For rotavirus, 120 days was sufficient to reduce the 95th percentile annual risk of infection for accidental ingestion to less than 10^{-4} at all temperatures (Fig. 4a–c). 115 days was sufficient for the consumption of lettuce (Fig. 4d–f). Increasing temperature decreased risks for rotavirus, similarly to *C. jejuni*. However, dilution had a greater impact on rotavirus decay, and in turn a greater impact on annual rotavirus risks. The lowest risks were observed for undiluted

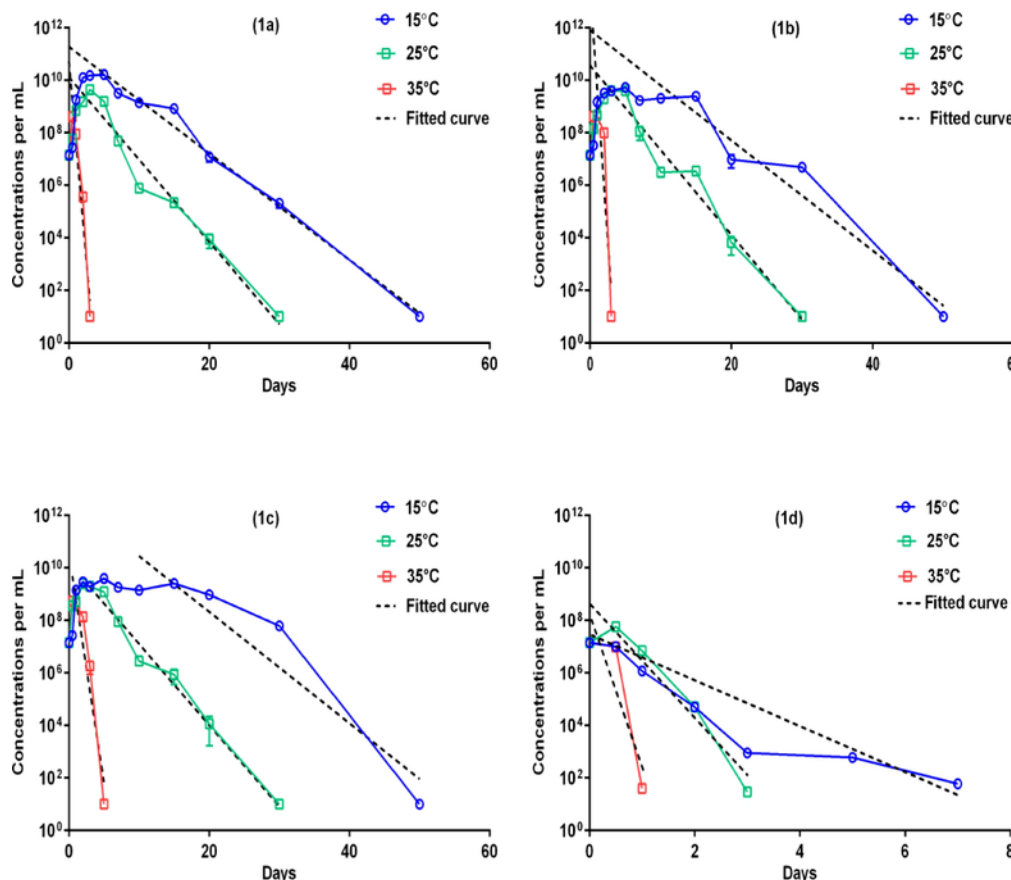


Fig. 1. Mean concentrations (CFU per mL) of *Escherichia coli* as a function of time (days) in (1a) undiluted; (1b) diluted 1:1; (1c) diluted 1:3; and (1d) stored urine samples at different temperatures. Standard deviations are shown, however, in most cases, the error bars are too small to illustrate. Dotted linear regression models fit to log transformed data represent regrowth period of *E. coli* (1–10 days).

urine compared to diluted urine. The difference in the effect of dilution was most apparent at 15 °C, where changing the dilution from 1:1 to 1:3 resulted in a difference of approximately 60 and 70 days of storage time necessary to meet a hypothetical risk benchmark of 10^{-4} for accidental ingestion and consumption of lettuce, respectively. At 25 °C, and 35 °C, the difference in annual risk between 1:1 and 1:3 dilutions was less than one order of magnitude.

A sensitivity analysis (Table 3) demonstrated that for the accidental ingestion route, the concentration of feces in urine (C_u) was the most influential predictor of the variability in annual risk for both *C. jejuni* and rotavirus (Spearman rank correlation coefficient range 0.671–0.681 for all temperatures). This was followed closely by the concentration of pathogens in feces ($C_{f,i}$) with Spearman rank correlation coefficients ranging from 0.645 to 0.653. The excretion duration (E_i) was the least influential parameter for accidental ingestion. For consumption of lettuce, the most influential parameters were the also the concentration of feces in urine and pathogen concentration in feces. The excretion duration played a comparable role to accidental ingestion. The in-field decay coefficient ($k_{f,i}$) played a larger role for *C. jejuni* (Spearman rank coefficient range -0.526 to -0.512) than for rotavirus (-0.056 to -0.041). The volume retained by lettuce (V_R) was a less influential parameter in both pathogens (*C. jejuni* 0.035–0.050; rotavirus 0.047–0.060).

4. Discussion

Knowledge on the persistence of the enteric pathogens in a urine storage tank is of importance for assessing the health risks related to the handling and use of the urine as fertilizer in agriculture. In this study, the inactivation of *E. coli* and MS2 phage was assessed in fresh human and source separated urine in sub-tropical Southeast Queensland, Australia for undiluted, diluted 1:1, diluted 1:3, and stored urine samples for three temperatures ranging from 15 to 35 °C. For inactivation experiments, *E. coli* and MS2 phage were chosen as representative pathogens because certain pathogens can be difficult to culture, assays can be expensive and may require a longer time to obtain results (Field and Samadpour, 2007). Several studies have evaluated the inactivation of fecal indicator bacteria and pathogens in stored urine as a function of time in mostly temperate regions (Höglund et al., 1998; Höglund and Stenström, 1999; Höglund, 2001; Höglund et al., 2002a; Vinnerås et al., 2008). To the best of our knowledge, this is the first study that reports the inactivation of *E. coli* (bacterial indicator) and MS2 (viral surrogate) in fresh human and stored urine in a sub-tropical country.

It has been reported that temperature, elevated pH, increased ammonia, and urine dilution can accelerate inactivation rates of *E. coli*, *Enterococcus faecalis*, *Salmonella* spp., and MS2 phage (Chandran et al., 2009; Höglund, 2001; Paruch et al., 2015; Vinnerås et al., 2008). Furthermore, urine is commonly stored in sealed containers to prevent ammonia-nitrogen loss and odor, resulting in a rapid conver-

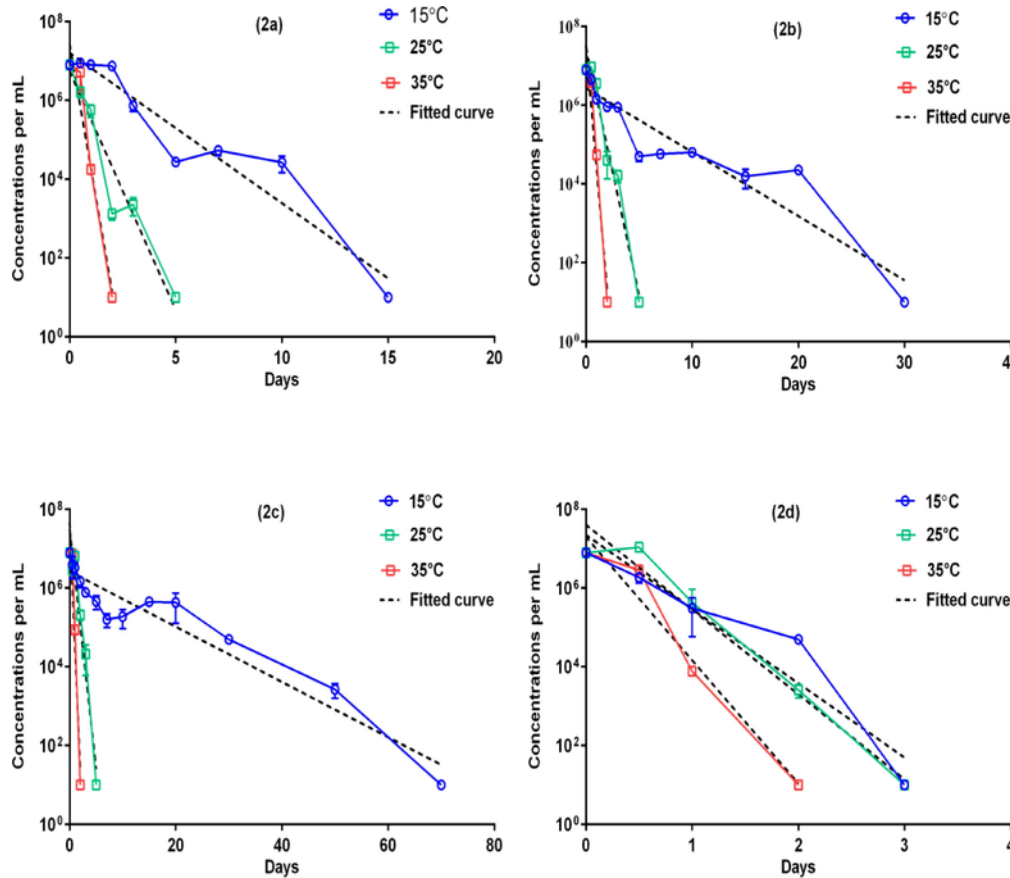


Fig. 2. Mean concentrations (PFU per mL) of MS2 phage as a function of time (days) in (2a) undiluted; (2b) diluted 1:1; (2c) diluted 1:3; and (2d) stored urine samples at different temperatures. Standard deviations are shown, however, in most cases, the error bars are too small to illustrate. Dotted lines show a linear regression model fit to the log transformed data.

Table 2

Inactivation rates (K) for *Escherichia coli* and MS2 phage in undiluted, diluted (1:1 and 1:3) and stored urine samples at different temperatures.

Temperature	Urine samples	<i>E. coli</i>		MS2	
		K	R^2	K	R^2
15 °C	Undiluted urine	0.20	0.98	0.38	0.92
	Diluted urine 1:1	0.21	0.93	0.16	0.86
	Diluted urine 1:3	0.21	0.87	0.07	0.89
	Stored urine	0.89	0.95	1.88	0.89
25 °C	Undiluted urine	0.31	0.95	1.21	0.91
	Diluted urine 1:1	0.32	0.94	1.25	0.96
	Diluted urine 1:3	0.31	0.97	1.22	0.95
	Stored urine	2.37	0.90	2.15	0.97
35 °C	Undiluted urine	3.04	0.94	3.14	0.94
	Diluted urine 1:1	3.83	0.75	3.10	0.94
	Diluted urine 1:3	1.75	0.90	3.14	0.91
	Stored urine	6.15	0.68	3.13	0.95

R^2 : Goodness of fit tests for the linear regression model

K : Inactivation rate per day

sion of urea to ammonia. This increases pH, which can also contribute to pathogen inactivation (Chandran et al., 2009; Vinnerås et al., 2008; WHO, 2006). In this study, *E. coli* and MS2 in fresh undiluted and diluted urine samples persisted longer at lower temperatures (15 and 25 °C) compared to high temperature (35 °C). The results of this study are in agreement with previous studies that reported slow inactivation of fecal indicator bacteria and pathogens in

urine samples at lower temperature (Chandran et al., 2009; Höglund et al., 1998; Vinnerås et al., 2008).

The inactivation rates of both *E. coli* and MS2 phage obtained in this study appear to be relatively higher than those previously published studies undertaken in Sweden and Norway (Chandran et al., 2009; Höglund, 2001; Paruch et al., 2015; Vinnerås et al., 2008). Several factors such as differences in pH of urine samples, background microorganisms of varying persistence rates, variations in experimental temperature between studies, types of seeding materials (strain vs. feces), starting concentrations of seeded microorganism, availability of nutrients in samples, and use of various inactivation models may have attributed to the higher inactivation rates. For example, in this study *E. coli* type strain was used as seeding material, whereas, Paruch et al., (2015) seeded urine samples with feces containing mixed *E. coli* populations. Chandran et al., (2009) incubated undiluted and diluted urine at 30 °C, whereas, in this study urine samples were incubated at 35 °C. Therefore, direct comparison of inactivation rates between studies is not straightforward.

Notably, *E. coli* regrowth occurred in diluted urine samples at all temperatures. The growth phases lasted longer (5–10 days) in the lower temperatures (15 and 25 °C) compared to a higher temperature (35 °C), where it lasted for one day. Davis et al., (2005) investigated the regrowth potential of six pathogenic *E. coli* strains in cedar chip bedding amended with 10% urine or 100% (undiluted) urine at 25 and 37 °C. For every strain, the addition of 10% or 100% urine to the bedding and incubation at 25 °C resulted in bacterial regrowth. Human urine can be hydrolysed to ammonia and carbon dioxide by ure-

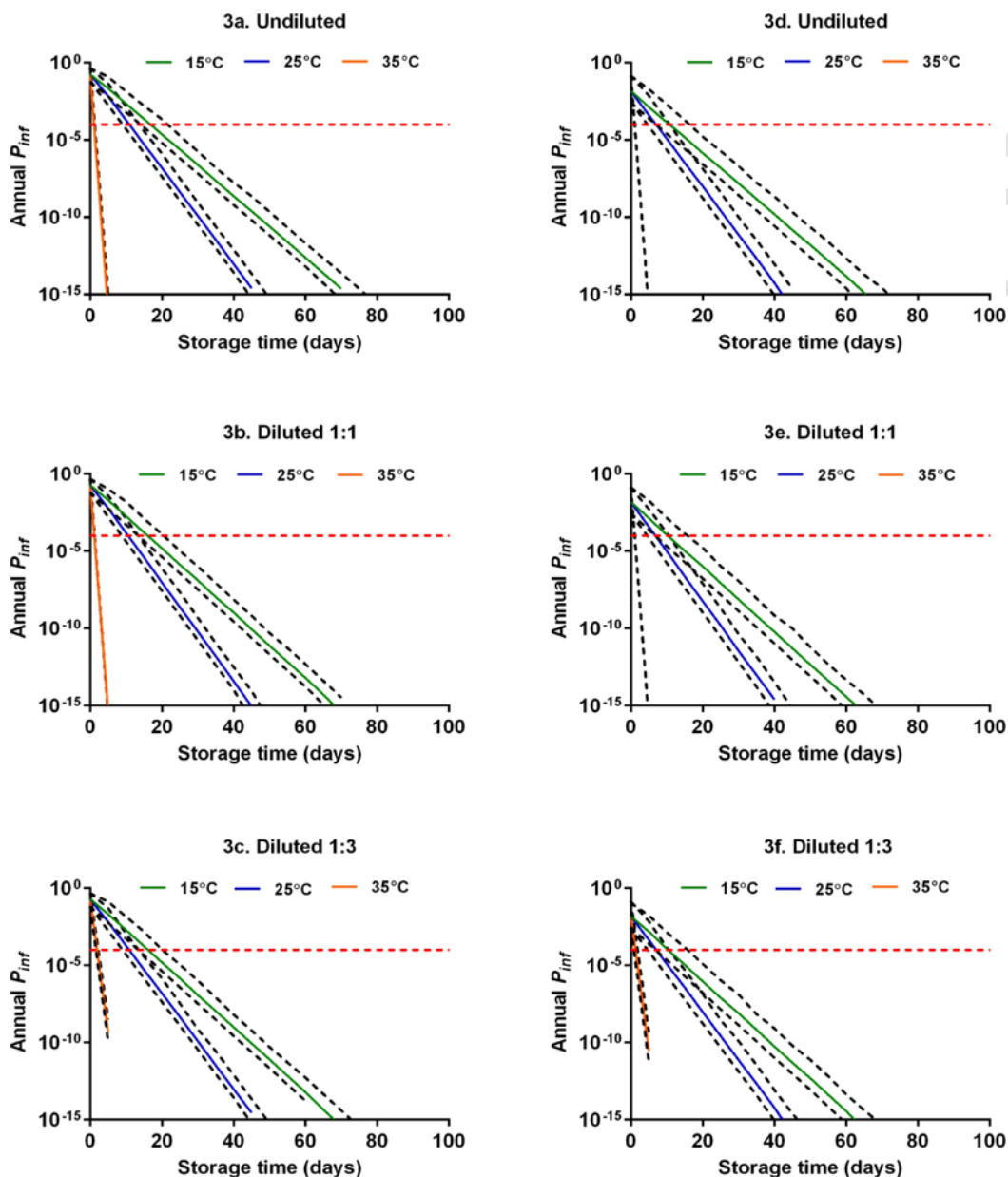


Fig. 3. Annual probability of *Campylobacter jejuni* infection for accidental ingestion of urine during irrigation (3a, 3b and 3c) and consumption of lettuce irrigated with stored urine (3d, 3e and 3f), simulated for various potential urine storage times. 95% confidence interval (5th and 95th percentile in black dashed line) of the median (solid line) shown for comparison with 10^{-4} annual infection reference point (horizontal red dashed line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ase. It is possible that the *E. coli* strain used in this study expressed urease activity in diluted urine samples at 15 and 25 °C. Alternatively, *E. coli* may be metabolizing unknown substances other than urea in urine (Roesch et al., 2003). The regrowth was observed for diluted urine samples but not for undiluted samples. It has been reported that regrowth of bacteria may occur in the presence of water (Austin, 2001). Added water to the fresh urine samples may have contributed the regrowth of *E. coli* in diluted samples.

The results of this study indicated that the inactivation rates (K) of *E. coli* and MS2 were higher for the six months aged stored urine samples. Therefore, the assumption of a linear decay rate over time may not be adequate. In the stored urine samples, urea and other organic substances are degraded by bacteria. The results are high ammonia concentrations, a high pH value and a pungent smell caused by

organic degradation products (Udert et al., 2003,2015). In this study, the pH of the stored urine samples had a high initial value of 9.0. Most microorganisms are adapted to a neutral pH, and highly acidic or alkaline conditions will have an adverse impact on their persistence (Cotter and Hill, 2003; Kazama and Otaki, 2011). The results obtained in this study are in agreement with previous studies that elevated pH level can rapidly inactivate *E. coli*, *Salmonella* spp., *E. faecalis* and MS2 phage (Chandran et al., 2009; Schöningg et al., 2002; Vinnerås et al., 2008). The high pH level may have also influenced the persistence of tested microorganisms in undiluted and diluted urine samples. When undiluted and diluted urine samples were stored at various temperatures, the pH increased to 8.5 to 9 (caused by microbial ureases) in 8–9 weeks and inactivation of seeded microorganisms was occurring and completely inactivated within 70 days.

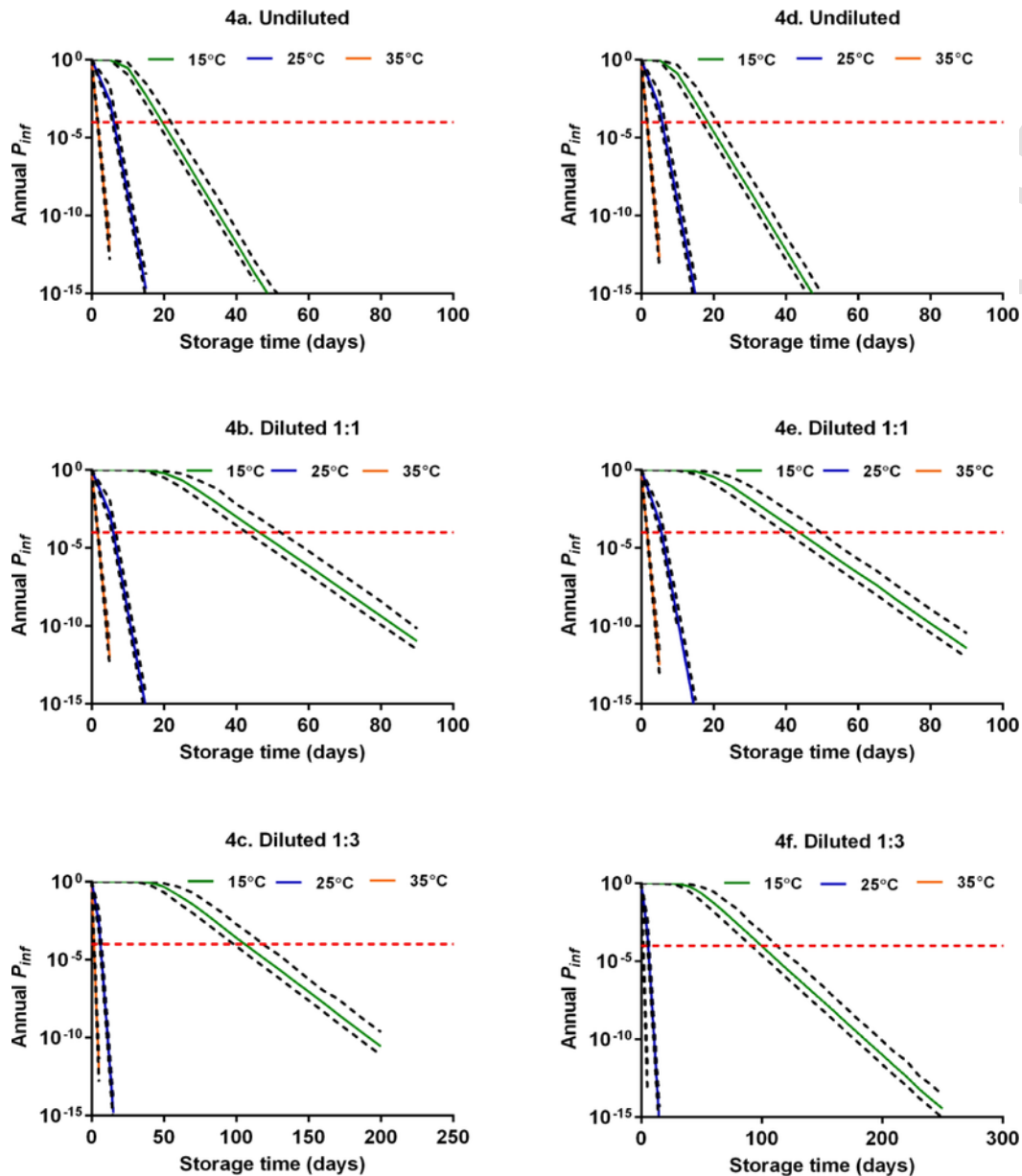


Fig. 4. Annual probability of rotaviruses infection for accidental ingestion of urine during irrigation (4a, 4b and 4c) and consumption of lettuce irrigated with stored urine (4d, 4e and 4f), simulated for various potential urine storage times. 95% confidence interval (5th and 95th percentile in black dashed line) of the median (solid line) shown for comparison with 10^{-4} annual infection reference point (horizontal red dashed line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Based on the fecal indicator bacteria and pathogen inactivation in urine samples, it has been recommended that urine must be stored for at least six months prior to its application as fertilizer for crop production (Höglund et al., 2002b; Jönsson et al., 1999). This time period has been considered sufficient for the reduction of pathogens especially viruses to a safe level in temperate regions (Höglund et al., 2002b). The simulated QMRA results obtained in this study indicated that 120 days (four months) of storage time would be appropriate for the inactivation of bacteria and viruses in a sub-tropical country if a 95th percentile value is compared to a hypothetical 10^{-4} annual risk benchmark. It is acknowledged that this benchmark was derived from drinking water, and, therefore most likely represents a conservative comparison point here.

Several limitations of the QMRA must be considered when interpreting these results (Table 5). The surrogate microorganisms used were considered to have the same characteristics as pathogens, and to persist similarly. As information was not available regarding their viability and/or infectivity, the measured concentrations are assumed to represent all available microorganisms with the ability to cause disease. Furthermore, it is assumed that the dose response curves for *C. jejuni* and rotavirus can be applied, that infectivity for the underlying animal models is representative of that for humans, and that there was minimal heterogeneity in human host susceptibility to the target pathogens.

Variability in the decay parameter K in stored urine was not considered. Additional experimental work to determine variability in K is recommended, especially as other studies have noted that pathogen

Table 3
Sensitivity analysis for annual risks.

Spearman rank correlation coefficient for Monte Carlo parameters (15 °C, 25 °C, 35 °C)					
Monte Carlo parameter	C_{fi}	E_i	C_u	k_{fi}	V_R
Scenario					
Accidental ingestion					
<i>C. jejuni</i>					
Undiluted	(0.648, 0.646, 0.645)	(0.262, 0.273, 0.273)	(0.679, 0.675, 0.678)	–	–
Diluted 1:1	(0.645, 0.643, 0.648)	(0.266, 0.268, 0.269)	(0.675, 0.683, 0.674)	–	–
Diluted 1:3	(0.651, 0.648, 0.647)	(0.268, 0.265, 0.265)	(0.676, 0.671, 0.678)	–	–
Stored	(0.649, 0.642, 0.641)	(0.277, 0.269, 0.268)	(0.674, 0.679, 0.676)	–	–
Rotavirus					
Undiluted	(0.652, 0.646, 0.651)	(0.245, 0.250, 0.252)	(0.678, 0.678, 0.677)	–	–
Diluted 1:1	(0.652, 0.651, 0.653)	(0.247, 0.247, 0.239)	(0.678, 0.681, 0.677)	–	–
Diluted 1:3	(0.654, 0.649, 0.650)	(0.242, 0.252, 0.245)	(0.680, 0.681, 0.681)	–	–
Stored	(0.651, 0.650, 0.649)	(0.245, 0.241, 0.250)	(0.678, 0.676, 0.680)	–	–
Consumption of lettuce					
<i>C. jejuni</i>					
Undiluted	(0.544, 0.538, 0.537)	(0.230, 0.232, 0.227)	(0.563, 0.570, 0.568)	(−0.524, −0.512, −0.519)	(0.035, 0.040, 0.045)
Diluted 1:1	(0.540, 0.540, 0.544)	(0.222, 0.227, 0.232)	(0.563, 0.564, 0.566)	(−0.524, −0.520, −0.524)	(0.040, 0.050, 0.050)
Diluted 1:3	(0.535, 0.534, 0.544)	(0.212, 0.231, 0.230)	(0.570, 0.567, 0.569)	(−0.522, −0.520, −0.518)	(0.046, 0.042, 0.041)
Stored	(0.539, 0.539, 0.541)	(0.221, 0.223, 0.230)	(0.568, 0.569, 0.569)	(−0.526, −0.519, −0.518)	(0.036, 0.044, 0.047)
Rotavirus					
Undiluted	(0.643, 0.654, 0.647)	(0.249, 0.250, 0.247)	(0.680, 0.677, 0.681)	(−0.050, −0.056, −0.041)	(0.048, 0.055, 0.054)
Diluted 1:1	(0.646, 0.651, 0.647)	(0.244, 0.245, 0.254)	(0.681, 0.675, 0.678)	(−0.054, −0.052, −0.051)	(0.049, 0.055, 0.047)
Diluted 1:3	(0.645, 0.651, 0.650)	(0.240, 0.251, 0.244)	(0.678, 0.675, 0.679)	(−0.050, −0.055, −0.044)	(0.060, 0.051, 0.053)
Stored	(0.652, 0.647, 0.647)	(0.248, 0.248, 0.245)	(0.680, 0.671, 0.675)	(−0.056, −0.048, −0.047)	(0.055, 0.051, 0.054)

Not applicable to the Monte Carlo simulation for the model indicated.

inactivation on crops largely influences the risk for this scenario (Höglund et al., 2002a; Petterson et al., 2001). Yearly incidence rates for disease previously modeled by Petterson et al. (2016) were used in the current QMRA model. This represents a sporadic infection scenario rather than a post-epidemic urine storage scenario over a short time period. If urine were stored directly following an outbreak of gastrointestinal disease, larger storage times are likely to be warranted between that event and the following irrigation period. This could also impact the concentration of feces cross-contamination in urine as the density of feces during diarrhea events is likely to be less

dense, and therefore more likely to partition into the liquid fraction of urine during a fecal contamination event. The concentration of feces in urine is difficult to determine in an individual toilet flush, and has been only determined in centralized storage tanks by using chemical fecal markers (Schönning et al., 2002). The time to fill the storage bladder is approximately one month before it is transferred to a centralized storage location (Beal et al., 2008). During this time, urine will enter the bladder in an incremental fashion, and inactivation rates may vary over time. The centralized storage location will receive urine from multiple households, further complicating the elucidation of the effect of multiple factors on an aggregate inactivation rate value.

For the lettuce consumption scenario, a short withholding period of two days was considered as in a previous model by Barker et al. (2013), due to the fact that in hotter Queensland meteorological conditions, there is likely to be a shorter withholding time because the plants are unlikely to thrive for more than two days without irrigation in warm weather. The volume of water retained by lettuce is likely to be conservative, as the information from Shuval et al. (1997) are for overhead irrigation, which may not be used for urine irrigation. However, in all cases, accidental ingestion risks were higher and would drive the storage time guidelines. Although storage time in the primary risk management intervention considered in this work, the storage time necessary to meet the acceptable target risk could be reduced by applying additional interventions to reduce pathogen exposure (Table 4). The impact of these other management interventions has not been quantified here but warrants further investigation in order to reduce storage times necessary to meet risk targets. These log reductions could be incorporated into the model through another factor of $10^{-\log\text{reduction}}$ from Table 3.

Since there was regrowth of *E. coli* at 15 and 25 °C, storage time reported in this study for *C. jejuni* should be interpreted with care. It was assumed that the initial regrowth phase observed for *E. coli*

Table 4
Intervention measures that can reduce pathogen exposure during source-separated urine use.

Intervention to reduce risk from urine	Descriptions	Log reduction (R) associated with interventions
Source control	Measures taken at the toilet including wiping from front to back, excluding young children and those with illness from using the UST. Urine used is sourced from the one household.	1 Log ₁₀ reduction in pathogen concentration in urine.
<i>When used to irrigate crops</i>		
Dilution of stored urine	Collected urine is diluted 1:10 parts urine to parts water to reduce foliage burning and odor (Joensson et al., 2004; Tilley et al., 2014).	1 Log ₁₀ reduction in pathogen concentration if diluted 1:10.
Withholding period	Urine is not applied to crops within a certain period of time before harvesting. A withholding period of 1 month between the last urine application and the harvest is recommended (WHO, 2006).	Reduction in pathogen concentration in urine varies as function of time of withholding and weather conditions. 2–3 log reduction expected.
Rinsing of produce	Produce such as lettuce are vigorously washed under tap water. Cooking or peeling fruits/vegetables is another effective measures to reduce the health risks (WHO, 2006).	Rinsing of salad crops such as lettuce to reduce bacteria by at least 1 log unit. Cooking food thoroughly would reduce pathogens by 2–6 log units.
Wearing protective clothing when handling urine	Wearing gloves and face masks, and using proper handling tools. Washing hands with soap after urine handling (WHO, 2006).	2 to 4 log reduction in pathogen concentrations if such interventions are taken.

Table 5

List of assumptions associated with the quantitative microbial risk assessment (QMRA) model.

Assumption
• Urine is used from a centralized storage tank only, not from individual household storage units. • Pathogens distributed homogeneously in well-mixed liquid fraction of centralized urine storage tank; significant additional contamination does not result from resuspension of sediments in storage tank. • Pathogens homogeneously distributed in contaminated feces. • Decay does not occur incrementally with flush inputs. • Linear decay assumptions assumed to hold over simulated urine storage timescales. • Surrogate pathogens (<i>E. coli</i>, MS2) are sufficiently representative of persistence of target index pathogens (<i>C. jejuni</i>, rotavirus). • Microbiological units from studies reported in literature used to derive microbiological input distributions of pathogens in feces are equivalent to each other and to those used in this study (1 PFU or 1 CFU = 1 pathogen). • Infectivity for animal models representative of that for humans. • No heterogeneity in human host susceptibility to target pathogens. • Shorter withholding period after irrigation before harvest in subtropical regions. • Urine irrigation does not occur directly after an epidemic period. • Overhead irrigation method applied. • Urine not treated in any way other than modifying urine storage time prior to application to crops. • Number of irrigation events per year is same for residential and occupational populations due to hot temperatures in subtropical Australia necessitating frequent irrigation. • Post-harvest removal mechanisms not considered.

would not occur under field conditions for bacterial pathogens, particularly for undiluted urine. However, if regrowth occurs under field conditions, the storage time presented here may not be sufficiently conservative for bacterial pathogens, especially for the duration of the regrowth phases. On the other hand, the concentrations of pathogens could be much lower in source-separated urine compared to the concentrations of *E. coli* and MS2 seeded in urine samples. In such scenario, a much shorter storage time may be required to achieve the effective removal of pathogens than the estimated storage time recommended in this study.

The persistence of microorganism in stored urine can be complex and affected by various factors such as temperature, dilution, presence of indigenous microorganisms, pH, and ammonia. Recreating realistic combinations of factors under laboratory conditions can be difficult. For example, stored urine samples are exposed to harsh environmental conditions with diurnal varying temperature compared to laboratory study where urine samples are incubated at a constant temperature. The inactivation of microorganisms needs to be investigated under field settings at ambient climatic conditions to obtain more accurate information on the inactivation patterns. It is likely that pathogens will inactivate more rapidly in field settings due to exposure to harsh climatic conditions such as high temperature in a subtropical region. In addition, the inactivation patterns of pathogens such as *C. jejuni*, rotavirus, adenovirus, and *Cryptosporidium parvum* should be investigated because these microorganisms may have different inactivation rates compared to fecal indicators such as *E. coli* and MS2 phage tested in this study (Ahmed et al., 2014b; Zhang et al., 2015).

5. Conclusions

- In summary, the results of this study suggest that high temperature influences the inactivation of microorganisms in urine, especially at temperature > 25 °C. Based on the results, it is recommended that urine storage tanks should be exposed to sub-tropical climates for rapid inactivation of pathogens. In addition, high initial pH appears to be another factor which may accelerate the inactivation of *E. coli* and MS2 phage in stored urine compared to fresh and diluted urine samples.
- Based on QMRA results, it can be concluded that source separated urine should be kept in the storage tank for approximately four months during cooler temperatures (15 °C) based on a comparison of simulated 95th percentile risks for accidental ingestion and consumption of irrigation of lettuce with a 10^{-4} annual risk benchmark, if no additional managements options are implemented. For warmer subtropical temperatures (25–35 °C), 15 days would be sufficient.
- Combining a QMRA-based approach to urine storage time with other pathogen reduction interventions as presented in this analysis provides a range of management options for UST users, and may reduce barriers to UST feasibility associated with long storage times. Accordingly, microbial health risks associated with the handling of urine in agriculture can be minimized to levels consistent with risk targets used for the Australian Recycling Water Guidelines (NRMMC-EPHC-AHMC, 2006).

Uncited reference

Gerba et al. (1996b).

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