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

W. Ahmed[a,*], K.A. Hamilton[a,b], A. Vieritz[c], D. Powell[d], A. Goonetilleke[e], M.T. Hamilton[f], T. Gardner[g]

[a] CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Qld 4102, Australia
[b] Drexel University, 3141 Chestnut Street, Philadelphia, PA 19104, USA
[c] Department of Science, Information Technology, and Innovation, Ecosciences Precinct, 41 Boggo Road, Qld 4102, Australia
[d] Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore, DC, Qld 4558, Australia
[e] Science and Engineering Faculty, Queensland University of Technology (QUT), GPO Box 2434, Brisbane 4001, Australia
[f] Yale University, New Haven, CT 06520, USA
[g] School of Medical and Applied Sciences, University of Central Queensland, Bruce Highway, Rockhampton, Qld 4702, 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 samples 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.

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 nitrogen, 50–65% of phosphorus, and 50–85% of 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 needed 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 in urine 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

* Corresponding author. Fax. +617 3833 5503.
E-mail address: warish.ahmed@csiro.au (W. Ahmed).

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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-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 for at least 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 regulatory concern in relation to 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, absence of specialized moving parts, and compatibility with Australian plumbing fittings. The toilets are connected to 300 L polyethylene storage tanks via 50 mm polyethylene pipes. The volume of flush water entering the storage tank 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 tank, 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 tank 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 Class 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 mixed 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 Millipore 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 (±1°), 25 °C (±1 °C), and 35 °C (±1 °C) 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