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Transfer Rates of Enteric Microorganisms in Recycled Water during Machine Clothes Washing[▽]

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Approximately 15% of overall Australian household water usage is in the laundry; hence, a significant reduction in household drinking water demand could be achieved if potable-quality water used for clothes washing is replaced with recycled water. To investigate the microbiological safety of using recycled water in washing machines, bacteriophages MS-2 and PRD-1, *Escherichia coli*, and *Cryptosporidium parvum* oocysts were used in a series of experiments to investigate the transfer efficiency of enteric microorganisms from washing machine water to objects including hands, environmental surfaces, air, and fabric swatches. By determining the transference efficiency, it is possible to estimate the numbers of microorganisms that the user will be exposed to if recycled water with various levels of residual microorganisms is used in washing machines. Results, expressed as transfer rates to a given surface area per object, showed that the mean transfer efficiency of *E. coli*, bacteriophages MS-2 and PRD-1, and *C. parvum* oocysts from seeded water to fabric swatches ranged from 0.001% to 0.090%. Greatest exposure to microorganisms occurred through direct contact of hands with seeded water and via hand contact with contaminated fabric swatches. No microorganisms were detected in the air samples during the washing machine spin cycle, and transfer rates of bacteriophages from water to environmental surfaces were 100-fold less than from water directly to hands. Findings from this study provide relevant information that can be used to refine regulations governing recycled water and to allay public concerns about the use of recycled water.

Approximately 15% of overall household water in Australia is used in the laundry (3); thus, a significant reduction in household drinking water demand could be achieved if drinking water used for clothes washing is replaced with recycled water. In addition, use of recycled water for clothes washing, a year-round activity, would provide a means to even out demand for recycled water throughout the year in existing dual reticulation schemes, where drinking water of a high quality and recycled water of a lower quality are delivered to households via separate pipes. Currently, in Australian dual reticulation schemes, recycled water is used for nondrinking purposes such as garden irrigation and toilet flushing, and there is higher demand for recycled water during hotter (and generally drier) summer months due to increased outdoor watering.

Public perceptions of potential health risks associated with contact with recycled water during laundry activities have the potential to restrict such use. Hence, it is important that assessments be performed to quantify the health risks associated with recycled water use for machine clothes-washing purposes. This information can be used in the development of recycled water regulations and to allay public concerns about the use of recycled water.

The Australian Guidelines for Water Recycling (Phase 1) released in December 2006 acknowledge the importance of recycled water to address water shortages and provide guidance on how such recycling can be safely and sustainably achieved for nondrinking uses (9). From a health perspective, guidelines focus on microbial hazards and use a quantitative microbial risk assessment (QMRA) process for guideline setting. QMRA consists of four steps, one of which is exposure assessment (15). Exposure assessment describes the conditions conducive to human exposure and typically includes a description of the intensity, frequency, and duration of exposure as well as of the exposure routes and the people exposed (15). Information required for QMRA modeling in relation to the use of recycled water for domestic laundry purposes includes data about the transfer rates of microorganisms from recycled water to hands, environmental surfaces, air, and fabric swatches. However, there is limited available data quantifying the transfer of microorganisms during laundry activities. Many of the published studies have been focused on laundering practices in hospitals (10, 19, 21, 23) and the communal environment (18), where infectious disease outbreaks can occur and where the role of soiled linen in disease transmission is of particular interest. Fewer studies have explored microbial transfer efficiencies specifically in the domestic situation and considered microbial transfer from fabric to fabric (12) or from fabric to hands (14, 20). However, even for these studies, much of the available information concerns the transfer of microorganisms from soiled wash items to environmental surfaces and hands and not from water to the fabrics being washed and to hands. More generally, the scarcity of information about the significance of fomites (in this context, environmental surfaces and fabrics, e.g.) in the spread of enteric disease means that better quantitative data including transfer rates on and from fomites are required to gain an understanding of the ecology of fomites in microbial transmission (6).

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To provide information to assist in the exposure assessment component of QMRA for use of recycled water for machine clothes washing, this series of experiments quantified the transference of microorganisms from washing machine water via multiple exposure routes to the person performing machine clothes washing. Identified microbial pathogen exposure routes included the following: (i) from water directly to the hands of the user, (ii) from water to the hands of the user following contact with contaminated fabric, (iii) from contact with environmental surfaces such as benches and washing machines, and (iv) via inhalation of aerosols generated during the washing machine cycle and/or discharge of spent water from the washing machine. Experimentation was confined to investigation of the transfer of enteric microorganisms. The rationale for this was that human dose-response data are available for ingestion of enteric pathogens but are not available for pathogens spread via dermal and inhalation routes. In addition, the results from this series of experiments were compared with exposure estimates used in the Australian Guidelines for Water Recycling, which focus on ingestion exposure to enteric pathogens during recycled water use.

MATERIALS AND METHODS

Design of the study. This study focused on determining exposure to selected microorganisms from recycled water to laundered clothing, the laundry environment, and directly to the hands of users performing household machine clothes washing. The transfer efficiency of microorganisms was determined using seeded waters for rinsing in a typical washing machine cycle. The selected microorganisms were chosen to represent different classes of human pathogens as follows: *Escherichia coli* to represent enteric bacterial pathogens, MS-2 bacteriophage to represent enteric viruses such as enterovirus and norovirus (16), and bacteriophage PRD-1 to represent viruses of a similar size such as rotavirus and adenovirus (24). As no satisfactory indicator organism has been identified for protozoan pathogens, gamma-irradiated (killed) *Cryptosporidium parvum* oocysts were used in experiments.

Experiments were designed to represent maximum exposure conditions that might occur during machine clothes washing. Top-loading washing machines (83% prevalence in Australian households) and cold water (used by 76% of households) were used, as this combination represents typical Australian practices (8). No detergents or other additives (sanitizers or fabric softeners) were used in the experiments.

A total of 21 separate experiments were conducted. Experiments using seeded water were conducted on eight occasions using *E. coli*, on six occasions using MS-2, on six occasions using PRD-1, and on one occasion using *C. parvum* oocysts.

Experimental setup. Experiments were conducted at the Commonwealth Scientific and Industrial Research Organisation laboratories in Highett, Victoria, Australia. The washing machine and stainless steel wash trough used for experiments were located within a "laundry" room of 10.7 m^3 within a physical containment level 2 (PC-2) laboratory facility. The dosing pump used for inoculating microorganisms and the reservoir holding the base water used in experiments were located outside of the laundry room within the main PC-2 laboratory. The laundry room was able to be closed off from the rest of the PC-2 laboratory, with entry to the room only during microbiological sampling events.

A Fisher and Paykel model GW 512 top-loading washing machine was used for experiments and was chosen as it is fully programmable with the capability to adapt the wash action, water levels, and the spin speed and to start from any point in the washing cycle (e.g., rinse and spin only). The rated capacity of model GW 512 is 5.5 kg of washing, and the volume of water for fill to the high level is 69 liters. The wash options selected for experimentation comprised the rinse (16-min duration) and spin (10-min duration) options only. A dosing pump (Gamma/4-W0803PP; Prominent Fluid Controls, Heidelberg, Germany) was used to dose microorganisms into the inlet line of the washing machine. Mixing the inoculum in the seeded water thus occurred prior to the entry of seeded water at the top of the washing machine tub. Further mixing occurred as the tub was progressively filled to the "high" tub level. The mean duration time of dosing was 4 min and 14 s ± 35%, while the mean time to fill the washing machine was 5 min and 1 s ± 43%.

Following each microbial seeding experiment, the washing machine and pipe work were disinfected using sodium hypochlorite solution. The presence of at least 5 ppm of free chlorine in the disinfected water in the washing machine tub was confirmed using BDH diethyl-*p*-diamine (comparator) number 1 tablets (VWR International Ltd., Lutterworth, United Kingdom). To ensure that residual chlorine was not present in the inlet pipe work or in the washing machine before subsequent experiments were conducted, 500 ml of sodium thiosulfate solution (10%) was dosed into the washing machine. Once the washing machine was filled and prior to commencement of the spin cycle of the washing machine, the absence of free residual chlorine in the water in the tub was confirmed.

Fabric swatches. The types of fabric selected for this study are commonly used for making garments and towels and would constitute the types of fabric that typically predominate in washing machine loads. Three types of dye-fast fabrics (locally purchased) were used. One fabric type was 65% polyester and 35% cotton (poly-cotton) of a knit weave. The other two types were 100% cotton; one of these sample types was toweling, and the other was a knit weave. The fabrics were initially washed with laundry detergent (Omomatic; Unilever Australia, Ltd) without bleach to remove any chemical residues from the fabric manufacturing process, rinsed several times in water, and dried in a laundry drier (21). Fabric swatches used in experiments were 100 cm^2 (10 cm by 10 cm). Prior to use in experiments, fabric swatches were sterilized by autoclaving at 121°C for 16 min, with an additional drying cycle (10 to 20 min). The mean weight of fabric swatches was 3.89 g (standard deviation, 0.17 g) for cotton toweling, 1.50 g (standard deviation, 0.06g) for poly-cotton knit fabric, and 2.19 g (standard deviation, 0.07g) for cotton knit fabric. Each load of laundry consisted of 30 fabric swatches, consisting of 10 swatches of each fabric type.

Sampling. (i) Air. Air samples were collected prior to microbial dosing of the washing machine and during the period from the beginning to the end of the spin cycle. The sampler was positioned 65 to 70 cm from the washing machine at a height of 72 cm from floor level on a bench adjacent to the washing machine.

For *E. coli* determinations, air samples were collected using a Merck MAS-100 air sampler (Merck, Darmstadt, Germany). The air sampler was set at 1,000 liters (1 m^3). This was equivalent to an average sampling duration of 10 min. Prior to operation, the Merck MAS air sampler was fitted with a Levine eosin-methylene blue (Oxoid Ltd., Basingstoke, United Kingdom) agar plate.

For bacteriophage and *C. parvum* oocyst determinations, air samples were collected using a diaphragm vacuum pump ME2 (Vacuubrand; GmbH & Co. KG, Wertheim, Germany) connected to a Hudson disposable humidifier (Hudson RCI, NC). The vacuum pump was operated for 10 min at a rate of 2 m^3/h. Prior to operation, the Hudson sampler was loaded with 50 ml of microbial trapping fluid, consisting of 0.1% peptone (buffered peptone; Oxoid Ltd., Basingstoke, United Kingdom).

(ii) Environmental surfaces. Environmental surfaces comprising the inside of the lid of the washing machine, the rim of the washing machine, and the outside surface of the washing machine were sampled using 3 M Quick Swabs (3 M, St. Paul, MN) prior to commencement of each seeding experiment and prior to commencement of the spin cycle. Prior to all study activities and following environmental sampling, environmental surfaces were disinfected using 70% alcohol. Two samples were collected from each surface (a total of six samples per sampling event). A 20-cm^2 area of each sampling location was swabbed using a stainless steel template which was placed over the surface being swabbed.

(iii) Fabric swatches. Fabric swatches were sampled from the washing machine using alcohol-flamed tongs. After swatches were removed from the washing machine, they were placed into individual sterile stomacher bags to which was added 100 ml of diluent (Ringer's solution; Oxoid Ltd., Basingstoke, United Kingdom) for bacteriophage or of deionized water for *E. coli* determinations (for compatibility with *E. coli* assay method). Fabric swatches were processed for 1 min at 230 rpm in a Seward Stomacher 400 Circulator (Seward Ltd., Worthing, United Kingdom) and then aseptically squeezed within the stomacher bag to remove excess liquid. For *E. coli* assays, all of the extract solution was transferred to a Colilert-24 reagent bottle containing enzyme hydrolysable substrate reagent (Idexx Laboratories, Westbrook, ME). For bacteriophage and *C. parvum* assays, replicate (2, 4, 5) sample volumes of extract were analyzed.

To determine the recovery efficiency of microorganisms from fabric swatches, 0.1 ml of prepared inoculum was inoculated onto each of the swatch types. The recovery efficiency (percent) from each swatch type was determined as follows: (count on swatch divided by inoculum seed level) × 100. For toweling fabric, the recovery efficiency was 82% ± 14%, 78% ± 4%, 73% ± 4%, and 98% for *E. coli*, MS-2, PRD-1, and *C. parvum* oocysts, respectively. For poly-cotton knit fabric, the recovery efficiency was 105% ± 7%, 77% ± 8%, 63% ± 6%, and 80% for *E. coli*, MS-2, PRD-1 and *C. parvum* oocysts, respectively. For 100% cotton knit

fabric, the recovery efficiency was 115% ± 19%, 68% ± 6%, 59% ± 9%, and 54% for *E. coli*, MS-2, PRD-1, and *C. parvum* oocysts, respectively.

(iv) Hands. One or both hands were sampled before and following contact with inoculated water and fabric swatches. Prior to the performance of experiments, the hands of the researcher (one researcher for all experiments) contacting seeded water and/or fabric swatches contaminated with residual microorganisms were thoroughly inspected to ensure their freedom from any apparent damage to the skin. Prior to all study activities, a control wash was performed comprising the following steps: squirting both hands with 70% alcohol for 10 s, rubbing the alcohol over the hands for 15 s, washing hands in liquid soap for 30 s, rinsing for 15 s, and, finally, drying with paper towels. For hand sampling, prior to and following submersion of hands in the inoculated water, two separate swab samples were taken. One swab was rubbed over the upper surface, and the other swab was rubbed over the lower (palm) surface. Both swabs were rubbed over the area from the second knuckle to the tip of all fingers, including the area between the fingers (total area, approximately 30 cm^2). For hand sampling prior to and following the handling of contaminated fabric swatches, swabs were rubbed over the palm-side surface of the thumb and fingers (total area, approximately 15 cm^2). Each procedure was performed using 3 M Quick Swabs (3 M, St. Paul, MN). After the swabbing step, the swab was returned to the tube of letheen broth and refrigerated (4°C) prior to microbial analysis, which was performed within 2 h of sampling. Hands were decontaminated with 70% alcohol and then washed in liquid soap before being dried on paper towels.

Bacteriophage propagation and assay. Bacteriophage propagation and assays were performed using *Salmonella* enterica serovar Typhimurium WG49 (NCTC 12484) and *S. enterica serovar* Typhimurium LT-2 (ATCC 19585) as the hosts for MS-2 and PRD-1 bacteriophages, respectively (all cultures obtained from M. Storey, University of New South Wales). To prepare the 500-ml inoculum of bacteriophage for dosing into the washing machine, frozen 1.2-ml stock suspensions were thawed and diluted in one-fourth strength Ringer's solution (Oxoid Ltd., Basingstoke, United Kingdom).

Bacteriophages were assayed via the use of the double agar overlay method (1). A total of 5.0 to 5.5 ml of suspension, obtained by extracting residual bacteriophages from fabric swatches (100 ml) and air samples (50 ml), was assayed. Likewise, a total of 1.0 ml of suspension obtained from environmental and hand sampling was screened for the presence of bacteriophage.

***E. coli* propagation and assay.** One Bioball (BTF Precise Microbiology, North Ryde, Australia) containing 30 organisms of *E. coli* ATCC 25922 was used to begin an overnight culture in 100 ml of tryptone soya broth (Oxoid Ltd., Basingstoke, United Kingdom). This culture was then diluted in 0.1% peptone (bacteriological peptone; Oxoid Ltd., Basingstoke, United Kingdom) to prepare 500 ml of inoculum used to dose the washing machine.

Water samples and extract from fabric (each 100 ml) were assayed for *E. coli* using Colilert-24 substrate technology (Idexx Laboratories Inc., Westbrook, ME) coupled with the Quanti-tray/2000 (Idexx Laboratories Inc., ME) for most probable number (MPN) enumeration (2). Water samples of 100 ml were assayed. Triplicate samples were incubated at 37°C for 24 h. MPN was determined as MPN per 100 ml of water sample.

Environmental surfaces were assayed for *E. coli* using EC Petrifilm plates (3 M, St. Paul, MN). EC Petrifilm plates, hydrated with 1 ml of letheen broth (Difco Laboratories, Sparks, MD) were used as contact plates and incubated at 37°C for 24 h, at which time blue colonies were counted, giving rise to an *E. coli* count per 20 cm^2.

Hand swab samples were assayed for *E. coli* using EC Petrifilm plates (3 M, St. Paul, MN). Quickswabs (3 M, St. Paul, MN) were returned to the letheen broth immediately following swabbing and stored at 4°C prior to analysis. To assay for *E. coli*, excess liquid was squeezed from the swab by pressing it against the side of the tube. All liquid contained in the tube was then transferred and spread onto the surface of an EC Petrifilm (3 M, St. Paul, MN) plate. Plates were incubated at 37°C for 24 h, giving rise to an *E. coli* count per swabbed area.

Eosin-methylene blue (Oxoid Ltd., Basingstoke, United Kingdom) plates removed from the air sampler were incubated at 37°C for 24 h, at which time metallic green colonies were counted, giving rise to an *E. coli* count per extracted air volume.

***C. parvum* oocyst propagation and assay.** Five milliliters of gamma-irradiated *C. parvum* oocyst suspension (purified calf fecal specimen at 1×10^7/ml; batch numbers 171 to 33) was obtained from BTF (North Ryde, NSW, Australia). The suspension was diluted in one-fourth strength Ringer's solution (Oxoid Ltd., Basingstoke, United Kingdom) to prepare 500 ml of inoculum used to dose the washing machine. Prior to the dosing of the washing machine with *C. parvum* oocysts, 1 ml of the total 500 ml of suspension was removed for analysis. Serial 10-fold dilutions of the suspension were then prepared to quantify the number of *C. parvum* oocysts in the seed suspension.

Samples for *C. parvum* were collected in sterile 100-ml glass sample bottles. Replicate 1-ml volumes were subsampled and placed in capped sample containers specially prepared by BTF Pty Ltd. (North Ryde, NSW, Australia). Samples were transported on ice within 24 h to the laboratory where analyses were performed. A total of 800 μl of sample was examined using flow cytometry. Prior to analysis of samples, the diluents in which samples were suspended (one-fourth strength Ringer's solution; Oxoid Ltd., Basingstoke, United Kingdom) and letheen broth (Difco Laboratories, Sparks, MD) were seeded with approximately 100,000 oocysts and stained with a fluoroscein isothiocyanate-labeled anti-*Cryptosporidium* antibody to ascertain whether these diluents contained particles that might cause interference with the detection method. These stained samples were analyzed by flow cytometry, and a region was defined on a graph of green fluorescence and side scatter that enclosed the entire population of oocysts. The number of particles that fell within the oocyst region was recorded.

Data presentation. The average counts recovered from the environmental surfaces, hands, and fabric swatches were determined and then used to evaluate microbial transfer efficiency from water to object or object to object. The number of microorganisms per swatch was calculated by multiplying the count obtained in the assay volume by the relevant dilution factor (for *E. coli* this factor was 1, for bacteriophages the factor ranged from 20 to 200, and for *C. parvum* the factor was 125). Microbial counts for fabric swatches were standardized using the relevant percentage for recovery efficiency. Statistical analysis was performed through the use of Microsoft Office 2000, Excel spreadsheet analysis tools, and STATA, version 9 (Stata Corporation, TX).

The number of microorganisms recovered on the swatches at time interval T1 (before spin) and at T2 (after spin) was divided by the total number of microorganisms in the washing machine tub. To calculate transfer from water to hands, the mean number of recovered microorganisms from the hand surface area of 30 cm^2 was divided by the total number of microorganisms in the washing machine tub. For transfer from water to surfaces, the mean number of recovered microorganisms from the environmental surface area of 20 cm^2 was divided by the total number of microorganisms in the washing machine tub. The transfer efficiency from fabric swatches following the spin cycle to the palm-side surface of the hands was calculated as the number of microorganisms recovered from a 15-cm^2 surface area of the palm-side fingertips following contact with the swatch divided by the number of microorganisms recovered on the respective fabric swatches (per 100 cm^2) after the spin cycle. A Poisson regression was used to derive confidence intervals associated with the fraction of microorganisms transferred (water to fabric or fabric to hands, e.g.). Where the reciprocal of the degrees of freedom (1/df) Pearson was >1 (most occasions), a Poisson regression adjusted for overdispersion was used.

RESULTS

Transfer route: recycled water to fabric swatches. Table 1 gives microbial transference rates from seeded water to fabric swatches. Results are computed as the percentage of microorganisms seeded into the machine tub that are transferred to fabric swatches of surface area 100 cm^2. Both the mean transfer rates and the variability in these rates (95% confidence interval) are given (no confidence limits are given for *C. parvum* oocyst transfer as only one experiment was performed). Taking the mean values alone, these results show that transfer rates from washing machine water to fabric swatches both before and after the washing machine spin cycle for all microorganisms is greatest for cotton toweling. Transfer rates from washing machine water to poly-cotton knit and to cotton knit fabrics were similar but less than that for cotton toweling fabric. Both before and after the spin cycle, the mean percent transfer efficiency value for all fabrics was highest for *E. coli* or *C. parvum* followed by MS-2 and then PRD-1, the lowest. Even when the variability in transfer efficiencies, as indicated by the 95% confidence intervals, is considered, the difference in transfer rates between microorganisms was statistically significant ($P < 0.01$ in all cases except one). Prior to the spin cycle there was no significant difference ($P = 0.06$) between *E. coli* and MS-2 transfer rates to cotton knit fabric (both, mean transfer

TABLE 1. Transfer efficiency of bacteria, bacteriophage, and *C. parvum* from water to fabric swatches

Organism and type of object or swatch	Microbe count (mean $\log_{10}$ CFU or PFU)			% Transfer efficiency (95% confidence interval)[a]	
	Machine tub and on 100-cm^2 swatch at T0[b]	100-cm^2 swatch at T1	100-cm^2 swatch at T2	Before spin cycle	After spin cycle
E. coli					
Machine tub	4.53				
100% cotton toweling	0	1.47	1.23	0.09 (0.08–0.10)	0.05 (0.04–0.07)
Poly-cotton knit	0	0.93	0.63	0.03 (0.02–0.03)	0.01 (0.01–0.02)
100% cotton knit	0	0.89	0.65	0.02 (0.02–0.03)	0.01 (0.01–0.02)
MS-2					
Machine Tub	7.94				
100% cotton toweling	0	4.58	3.93	0.04 (0.04–0.05)	0.01 (0.007–0.01)
Poly-cotton knit	0	4.20	3.51	0.02 (0.016–0.02)	<0.01 (0.004) (0.003–0.005)
100% cotton knit	0	4.19	3.75	0.02 (0.016–0.02)	<0.01 (0.006) (0.005–0.008)
PRD-1					
Machine tub	7.75				
100% cotton toweling	0	4.20	3.46	0.03 (0.025–0.033)	<0.01 (0.005) (0.003–0.007)
Poly-cotton knit	0	3.87	2.89	0.01 (0.011–0.016)	<0.01 (0.001) (0.001–0.002)
100% cotton knit	0	3.89	3.26	0.01 (0.012–0.016)	<0.01 (0.003) (0.002–0.004)
C. parvum					
Machine tub	7.30				
100% cotton toweling	0	4.08	3.97	0.06	0.05
Poly-cotton knit	0	3.82	3.71	0.03	0.03
100% cotton knit	0	3.82	3.80	0.03	0.03

[a] The number of microorganisms in the washing machine tub has been used as the denominator to calculate transfer efficiency: (number of microorganisms recovered from the swatch/total number in washing machine tub water) × 100.
[b] T0, prior to seed dosing of tub.

of 0.02%); however, the transfer rate of PRD-1 to cotton knit fabric was significantly less than that of either *E. coli* or MS-2 ($P < 0.01$).

Table 2 shows the average numbers of microorganisms on fabric swatches following the washing machine spin cycle, expressed as a percentage of the numbers on fabric swatches before the spin cycle. These results show that retention rates on fabric swatches following the spin cycle from, highest to lowest percentage, was as follows: *C. parvum* > *E. coli* > MS-2 > PRD-1.

Transfer route: fabric swatches to hand. Table 3 shows the transfer results from contaminated fabric swatches to the surface area (15 cm^2) of hands (fingertips) likely to contact the fabric and then the lips. Transfer rates for *E. coli* and bacteriophage PRD-1 were able to be obtained only as semiquantitative estimates as neither organism was detected on swabbed hands following handling of the contaminated swatches. The transfer rate for bacteriophage MS-2 from contaminated fabric to hands was 0.19%, with a 95% confidence interval of 0.05 to 0.79%.

Transfer route: recycled water directly to hands. Table 4 shows the transfer rate of microorganisms from seeded water to hands. Results are computed as the percentages of microorganisms seeded into the machine tub that are transferred to the hands in a surface area of 30 cm^2 (both sides of the fingertips contact the water). Results show that mean transfer efficiency from water to hands is greatest for *E. coli* and least for bacteriophage MS-2. When the 95% confidence limits are considered, the transfer rate of *E. coli* from water directly to hands is significantly greater than for bacteriophage MS-2 ($P < 0.01$) but not for PRD-1 ($P = 0.242$).

Transfer route: recycled water directly to environmental surfaces. Table 5 shows the transfer rate of microorganisms from seeded water to environmental surfaces (surface area, 20

TABLE 2. Ratio of microbial swatch counts at T2 and T1

Swatch type	% Microbial retention on fabric swatches (T2 count/T1 count)[a]			
	E. coli	MS-2	PRD-1	*C. parvum*
100% cotton toweling	57.3	22.4	18.1	78.5
Poly-cotton knit	49.8	20.3	10.6	78.0
100% cotton knit	57.3	36.0	23.9	96.7

[a] T2, after spin cycle; T1, before spin cycle.

TABLE 3. Transfer efficiency from spun fabric swatches to hands

Organism	Microbe count (mean $\log_{10}$ CFU or PFU) on:		% Transfer efficiency (95% confidence interval)[a]
	Swatch after spin cycle	Hands (fingertips swabbed)	
E. coli	0.93	<1	<11.64
MS-2	3.77	1.01	0.19(0.05–0.79)
PRD-1	3.25	<1	<0.1

[a] Percent transfer efficiency to hands from swatch is calculated as follows: (number of microorganisms recovered from 15-cm^2 surface area of hands/the number of microorganisms recovered from the swatch at T2) × 100.

TABLE 4. Transfer efficiency from water to hands

Organism	Microbe count (mean $\log_{10}$ CFU or PFU) in/on:		% Transfer efficiency (95% confidence interval)[a]
	Washing machine tub water	Hands (per 30 cm^2)	
E. coli	4.53	−0.90	0.00037 (0.00009–0.0015)
MS-2	7.94	1.34	0.000025 (0.000016–0.00004)
PRD-1	7.75	1.46	0.000052 (0.000012–0.000023)

[a] Percent transfer efficiency to hands from water is calculated as follows: (number of microorganisms recovered from surface area of hands/number of microorganisms in the tub water) × 100.

TABLE 5. Results for water to surface transfer

Organism	Microbe count (mean $\log_{10}$ CFU or PFU) in/on:		% Transfer efficiency (95% confidence interval)[a]
	Washing machine tub water	20-cm^2 surface	
E. coli	4.53	<1	<0.003
MS-2	7.94	−0.33	0.0000005 (0.00000015–0.000002)
PRD-1	7.75	−0.52	0.0000004 (0.0000001–0.0000017)

[a] Percent transfer efficiency to environmental surfaces from water is calculated as follows: (number of microorganisms recovered from a swabbed area of a 20-cm^2 environmental surface/number of microorganisms in the tub water) × 100.

cm^2) surrounding the washing machine. Results are computed as the percentages of microorganisms seeded into the machine tub that are transferred to environmental surfaces. Results show that the transference rates from water to environmental surfaces are 100-fold less than from water directly to hands, based on transfer to an equivalent surface area.

DISCUSSION

The overall purpose of experiments in determining the transfer efficiencies of enteric microorganisms from water to air, environmental surfaces, hands, and fabrics was to assess the exposure to enteric microorganisms that occurs during a typical washing machine cycle. With this information and information about the frequency of washing machine use for clothes washing, QMRA can determine the minimum microbiological quality of water that can be supplied for washing machine use without presenting an unacceptable health risk to consumers.

This series of experiments was commenced prior to the release of the Australian Guidelines for Water Recycling in December 2006 and in advance of the use of a QMRA process for Australian recycled water guideline setting. As a result, the seeding levels of *E. coli* used in experiments, rather than being at a level that would allow the resolution of transfer efficiencies in all instances, were aligned with levels of *E. coli* permitted in various classes of recycled water, as defined in the Australian State recycled water regulations at the time. Therefore, for *E. coli* experiments, seed levels were used corresponding to class A (<10 *E. coli* per 100 ml) and class B (<100 *E. coli* per 100 ml) recycled water classifications. Only one experiment was conducted using water containing levels of *E. coli* corresponding to class A recycled water. Results for this experiment did not demonstrate the transfer of *E. coli* to fabric swatches, hands, environmental surfaces, or air; hence, subsequent experiments using this seeding level of *E. coli* were not performed. Seven experiments were conducted using water containing levels of *E. coli* corresponding to class B recycled water. In these experiments, transfer of *E. coli* to fabric swatches (Table 1) and directly to hands (Table 4) was able to be demonstrated, but no *E. coli* was detected on hands after contact with contaminated swatches (Table 3) or on environmental surfaces (Table 5) following the machine washing cycle. The failure to detect *E. coli* on hands after the contact with contaminated fabric, on environmental surfaces, and in air samples limited findings to a semiquantitative estimate rather than quantitative information needed for input into the QMRA process. Despite this limitation, results of these experiments using a low *E. coli* inoculum level of 100 *E. coli* per 100 ml nonetheless provide data that can be referenced when experiments using high inoculum levels are conducted. Both quantitative and semiquantitative estimates generated in these experiments can be used to verify whether transfer rates are similar in experiments using high inoculum levels.

In contrast to *E. coli* seeding levels, seeding levels for bacteriophages MS-2 and PRD-1 were sufficient to quantify the transfer of these viruses from seeded water to hands of the washing machine user (Table 4) and to environmental surfaces (Table 3). Seeding levels of *C. parvum* oocysts were also sufficient to demonstrate transfer rates to fabric swatches (Table 1); however, problems associated with assay methods employed to assess the number of oocysts transferred to hands and the air meant that no data were obtained for oocysts for these transmission routes. No transfer rates for water or from fabric swatches to hand were obtained for *C. parvum* oocysts as the suspending fluid (letheen broth) into which swabs were placed interfered with the *C. parvum* oocyst assay. The combination of the yellow of the letheen (swab) suspending medium, which interfered with the fluorescent detection system, and the similar size of particles collected by the swab and *C. parvum* oocysts are possible reasons for observed false-positive detections. Particles similar in size to *C. parvum* oocysts extracted from the air similarly interfered with the detection methodology. Accordingly, more experiments should be conducted using the same level of *C. parvum* oocysts but including improvements to assay methods to overcome the limitations of these experiments.

Taken together, experimental results provide information about the relative transference of different categories of microorganisms via multiple pathways during a machine clothes-washing cycle. Results for microbial transfer from water to fabric swatches (Table 1) show that before and after the spin cycle, the transfer efficiencies of *E. coli* and *C. parvum* oocysts to fabric are greater than for bacteriophages MS-2 and PRD-1 for all fabric types. These results indicate that microbial size is an important determinant in the fabric attachment-detachment process during the machine washing cycle, with larger microorganisms showing greater transference to, and retention on, fabric swatches than smaller ones. Results (Table 1) also

show that for all microorganisms, transfer efficiencies are greater for cotton toweling than for other fabric types both before and after the washing machine spin cycle, indicating that it is not only the properties of the microorganism that influence transfer efficiency but also the properties of the fabric. Higher transfer efficiencies to toweling fabric than for other fabrics are not surprising, given the greater absorbency of this fabric relative to other fabric types. Another factor that may affect greater transfer to the toweling fabric includes its surface character, with its coarse rather than smooth surface, offering greater potential for enmeshing microorganisms within the fabric matrix. Results expressing mean microbial counts on the swatch after the spin cycle as a percentage of organisms present on the swatch before the spin (Table 2) show microbial retention, from highest to lowest amounts, for all fabric types after the spin cycle to be as follows: *C. parvum* > *E. coli* > MS-2 > PRD-1. When these results are overlaid onto swatch-water weight ratios expressed in the same manner (25.1%, 24.9% and 46.6% for cotton toweling, poly-cotton, and cotton knit fabrics, respectively), it is evident that the numbers of *C. parvum* oocysts and *E. coli* organisms present on the swatches are in excess of predicted numbers based on water content. Percent retention of bacteriophage MS-2 and PRD-1 more closely mirrors that of water retained in the fabric following the spin cycle. Greatest reduction in fabric-associated bacteriophage following the spin cycle was noted for PRD-1. As PRD-1 is larger than MS-2, this observation indicates that factors such as hydrophobicity and electrostatic properties of microorganisms in addition to microbial size are also likely to be important to fabric retention.

Hydrophobicity has been implicated as a factor in microbial attachment to fabrics in prior experiments. Experiments performed using radioisotope-labeled bacterial suspensions to investigate the adherent behavior of gram-positive and gram-negative bacteria on cotton, polyester fabrics, and their blends (17) found that the attachment of bacteria to fabrics was dependent upon both the types of bacteria (including their hydrophobicity) and the physicochemical characteristics of the fabric substrates (for example, polyester is a hydrophobic polymer). Adherence of *E. coli* to cotton fabrics was found to gradually increase with contact time, whereas the extent of *E. coli* adherence on polyester leveled off at 4 h (17).

Results for the transfer of microorganisms from seeded swatches to hands showed that the transfer efficiency of MS-2 was 0.19% (Table 3). Due to low numbers of *E. coli* and PRD-1 organisms compared to MS-2 on fabric swatches following the spin cycle, transfers of *E. coli* and PRD-1 organisms were able to be estimated only semiquantitatively. As discussed above, the numbers of *E. coli* organisms seeded into the washing machine were lower than for *C. parvum* and bacteriophages, which meant that the transfer efficiency to hands was able to be determined only as <12%. Based on significantly higher numbers of PRD-1 than *E. coli* organisms on fabric swatches, transfer efficiency for PRD-1, while also semiquantitative, was able to be expressed with greater accuracy as <0.1%. In computing transfer efficiencies the assumptions were made that the surface area of the swatch (100 cm^2) was the area that might be contacted by the area of the hand (15 cm^2) and that the surface of the fingertips (palm side) that was swabbed was most likely to come in contact with the nose and/or the mouth. In reality, not all organisms on the swatch may have the potential to be transferred to the fingers because of the crumpling and folding of the fabric after the spin cycle; therefore, the calculated percent transfer may be an underestimate. The transfer efficiency obtained in this series of experiments from fabric to fingers of <0.1 to 0.19% is in accord with rates reported by other investigators for 100% cotton and poly-cotton (<0.01 to 0.13%) transfers (20).

A more direct means of exposure of the hands to recycled water during machine clothes washing is the direct contact of hands with the water when clothing is added or removed during the washing machine cycle. Results show that the mean transfer rate from recycled water to hands was greatest for *E. coli* and least for MS-2 (Table 4). The difference between *E. coli* and MS-2 transfer rates was statistically significant ($P < 0.01$), but this was not the case for *E. coli* and PRD-1 ($P = 0.242$), requiring further investigation. The numbers of microorganisms transferred from recycled water and retained on hands was approximately 100 to 1,000 times less than the numbers transferred to fabric swatches, based on transfer to an equivalent surface area.

The deposition of droplets containing pathogens on surfaces as a consequence of airborne dissemination of microbes following aeration of the water and/or from splashing has been the focus of other research studies (4, 5, 7, 13). This fallout is of concern since hand contact with contaminated surfaces may result in self-inoculation by touching of the nose and mouth. Results of these experiments showed that there was some transfer of microorganisms from seeded water to environmental surfaces. Transfer levels, however, were low (Table 5). Transfer to environmental surfaces was approximately 100-fold less than that from recycled water directly to hands.

Air sampling followed protocols used by other investigators (5); however, the length of time that the air sampler was operative was limited by the duration of the spin cycle of the washing machine. This meant that the total amount of air able to be sampled within this time limit (10 min) was 1,000 liters for *E. coli* and 333 liters for bacteriophage and *C. parvum oocysts*. All air samples were negative for target (*E. coli*, MS-2, and PRD-1) microorganisms.

The decision to express results as transfer rates to a given surface area per object was based on the likely surface areas of the fabric or environmental surface coming in contact with a hand during machine clothes washing or the area of fingertips likely to come into contact with the lip subsequent to clothes washing. Alternatively, transference data may have been expressed as per cm^2 or per gram of laundry and combined with the following types of information: the number of items per washing machine load, the weight of the washing machine load, the number of times the washer touches the washed load, the surface area of the load touched by the washer, the number of times the washer places fingertips in the water during the washing machine cycle, etc. These data inputs would enable further refinement of exposure volume estimates, but such data are not available.

This series of experiments differs from other experiments where microbial survival and/or transfer during machine clothes washing have been investigated. This is because the focus of other studies has been infection control and/or the transfer of microorganisms from soiled laundry to the washing

machine water and to the washing machine user (10, 12, 14, 22, 23). Reports in the scientific literature show that fecally soiled clothing can itself contribute to the concentration of organisms to which the water user may be exposed (10, 12, 18). The number of enteric pathogens transmitted from soiled clothing to wash water and from one laundry item to another may be significant. For example, investigators have determined that the average pair of underwear contains about 0.1 g of feces (11). Based on an infected individual excreting up to 10^{10} *Salmonella* bacteria per gram of feces, this means that a wash load containing a single contaminated garment would be starting out with 10^8 or 10^9 *Salmonella* bacteria (11) before the addition of recycled water.

In this study the focus of experimentation was not on the transmission of microbial pathogens from contaminated laundry to the user but from recycled water to the user (directly or indirectly). Thus, one of the primary considerations in experimental design was that the transfer of microorganisms from recycled water to the user was dissociated from microbial transfer from soiled laundry to the user. Consequently, the assumption was made that the initial washing procedure, which generally incorporates the use of washing detergents and sanitizers and which includes the evacuation of most water from the washing machine prior to entry of the rinse water, would result in a significant reduction in microorganism numbers. This is consistent with reports in the literature showing up to 99.99% (4 $\log_{10}$) removal of microorganisms with the use of a sanitizer (12, 14) and the importance of prewashing (10). Accordingly, the starting point of experimentation was clean (sterile) fabric swatches washed in drinking water (cold water) with no addition of washing detergent or sanitizers. While use of a detergent and sanitizer and evacuation of washing water prior to rinsing may not necessarily result in total removal of enteric microorganisms (depending upon the soiled washing inoculum), this was a realistic starting point for experiments, allowing the impact of rinse waters of different microbial qualities to be assessed independently of other factors.

Taking available information from these experiments for bacteriophages MS-2 and PRD-1 and converting the mean number of bacteriophages detected on hands after handling contaminated fabric swatches and following contact with seeded water (based on the numbers of bacteriophage in seeded washing machine water), the total volume exposure estimate to hands is 0.03 ml and 0.04 ml for MS-2 and PRD-1, respectively. Of note is that these volume estimates are based upon maximum exposure associated with a top-loading washing machine able to be accessed during the washing cycle, a cold water wash, and an absence of detergents or other laundry products. Use of a front-loading washing machine, hot water, and detergents and sanitizers would result in lower exposure. For the purpose of exposure assessment in relation to enteric pathogens, it is the ingestion volume of water containing residual pathogens that is important; hence, information is required about hand-to-mouth transfer of viruses to provide an ingestion volume estimate. There is some available information in the literature that provides needed data about the transfer of viruses from contaminated hands to the mouth (20); however, more data are required. By using the 33.90% transfer rate obtained by Rusin and other researchers (20) for hand-to-mouth transfer of PRD-1, the total volume estimate transfer to hands of 0.04 ml can be converted to an ingestion exposure volume estimate of approximately 0.01 ml. This volume estimate although a point estimate is equivalent to the inadvertent ingestion exposure volume estimate given in the Australian Guidelines for Water Recycling for machine clothes washing (9). Of note, however, is that these guidelines indicate that inadvertent ingestion is via sprays generated during the machine washing process. This contrasts with the results of these experiments, which indicate that direct contact with recycled water and with fabric washed in such water provides greater exposure than exposure via contamination of the surrounding environment and the air. Immersion of the hands and handling damp fabric may occur multiple times during laundering; however, it is likely that exposures occurring on a single day are not cumulative as microorganisms on the skin may be resuspended if hands are wet again, or the microorganisms may be wiped off if hands are dried on a towel.

The small number or the absence of microorganisms detected on hands, on environmental surfaces, and in air despite microbial seeding doses in excess of that which might be realistically encountered in high-quality recycled water underscores the relatively low risk that highly treated recycled water presents to the washing machine user. Levels of bacteriophage used in these experiments were 6 to 7 $\log_{10}$ higher than viral levels present in recycled water proposed for machine clothes washing. In addition, based on the potential for high numbers of pathogens present in the soiled laundry, there will be many instances where the numbers of enteric pathogens present in recycled water (if present at all) will comprise only a small percentage of those present on soiled laundry.

While there is potentially some scope to vary the level of treatment that may be applied to wastewater to produce water of a quality suitable for machine clothes washing, other factors need to be considered. For example, use of lower-quality recycled water may present a greater health risk associated with inadvertent ingestion and/or potential drinking water cross-contamination incidents. The suitability of recycled water for machine clothes washing will also depend on the physicochemical quality of the water. This is because the esthetic properties of the water (color and turbidity) will also affect public acceptance of recycled water for clothes washing.

In conclusion, data generated from this series of experiments provide the following: (i) evidence to support an inadvertent ingestion exposure volume of 0.01 ml for recycled water used for machine clothes washing for use in the QMRA process; (ii) methodological information about the conduct of similar experiments; and (iii) input data for QMRA processes where information about fabric-to-hand and water-to-hand microbial transfers is required, resulting in a narrowing of the data gaps and thereby assisting in the further refinement of exposure volume estimates. Areas of potential future research to provide more input into the QMRA process include additional experiments with *C. parvum* oocysts and high inoculum levels of *E. coli* to verify results of these experiments and investigation of the transfer of microorganisms from surfaces to hands, from hands to mouth, and by inhalation exposure. In addition, future research could encompass experiments evaluating the transfer of a combination of enteric microorganisms as well as the transfer of nonenteric pathogens during machine clothes washing. Observational and other studies are also re-

quired to provide inputs into QMRA models so that potential exposure can be refined and calculated over a year. These include studies investigating the number of washing machine cycles per household over an extended period and details of the machine washing process (i.e., load weight, items per load, surface area of load contacted, etc.) typically used by households.

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