Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Survival of *Escherichia coli* in common garden mulches spiked with synthetic greywater

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Significance and Impact of the Study: Potential for microbial contamination is one of the limiting factors for domestic greywater reuse. Although subsurface irrigation is considered to be one of the lowest risk applications, there is still a possibility of microbes reaching the soil surface if the environmental conditions are not favourable or if soil movement inadvertently exposes the irrigation line. In these circumstances, the soil cover layer may be contaminated by greywater microbes. This study assesses the survival rates of the pathogen indicator organism *Escherichia coli* in three soil cover materials commonly used worldwide and makes clear recommendations to facilitate the safe reuse of domestic greywater.

Keywords
Escherichia coli, greywater, irrigation, mulch, pathogen survival.

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2017/0173: received 25 January 2017, revised 1 March 2017 and accepted 2 March 2017

doi:10.1111/lam.12732

Abstract

Reuse of domestic wastewater is increasingly practiced as a means to address global demands on fresh water. Greywater is primarily reused via subsurface irrigation of gardens, where the soil environment is seen to be an integral part of the treatment process. The fate of biological contaminants (i.e. pathogens) in the soil is reasonably well understood, but their persistence and survival in soil cover layers is largely unexplored. This study investigated the ability of *Escherichia coli* to survive in common soil cover layers. Three garden mulches were investigated: pea straw mulch, a bark-based mulch and a coconut husk mulch. Each mulch was treated with an *E. coli* solution, a synthetic greywater with *E. coli*, or a freshwater control. *Escherichia coli* was applied at 1×10^4 most probable number (MPN) per g dry weight mulch. Subsamples were temporally analysed for *E. coli*. The bark and coconut husk mulches showed a steady decline in *E. coli* numbers, while *E. coli* increased in the pea straw mulch for the duration of the 50 days experiment, peaking at $1{\cdot}8 \times 10^8$ MPN per g dry weight mulch. This study highlighted the importance of selection of a suitable material for covering areas that are subsurface irrigated with greywater.

Introduction

An increasing population, urbanization, industrial agriculture practices, pollution and climate change are placing a cumulative demand on our freshwater supply. Global interest in recycling of domestic wastewater for non-potable uses is growing. Greywater represents the largest portion of domestic wastewater, and may account for 50–80% of total household water use (Christova-Boal *et al.* 1996). Greywater is household water from kitchen sinks, dishwashers, washing machines, showers, baths and basins (Nolde 2000). The quality and composition of greywater is dependent on the household from which it originates, and is influenced by factors such as occupant age, state of health, lifestyle and consumer choices (WHO, 2006). Faecal contaminants can be washed off in the bathroom sink, shower or washing machine. This allows pathogens to enter the greywater system and therefore provide a risk of infection to those who come into contact with the greywater, for example, following garden irrigation (Casanova *et al.* 2001; Ottosan and Stenström 2003). Greywater has been shown to contain multiple micro-organisms, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Shigella* spp., *Klebsiella pneumoniae* and *Salmonella enterica* (Winward *et al.* 2008; Benami *et al.* 2015). Although variable and highly dependent on the

household from which the greywater originates, the potential presence of such pathogens is a major impediment for greywater reuse (Benami *et al.* 2015). The survival of these pathogens in the environment could increase the potential for exposure and risk to human health.

Escherichia coli is commonly used as an indicator organism for the potential presence of pathogens in environmental samples (Baudišová 1997; Tallon *et al.* 2005). Despite its documented limitations, including the ability to survive and proliferate outside a primary host (Ishii *et al.* 2006; Brennan *et al.* 2010), the absence of a better alternative means that it is still the best option for monitoring of faecal pollution in the environment (Edberg *et al.* 2000). Several studies have demonstrated the persistence of *E. coli* or faecal coliforms in soils irrigated with greywater and/or wastewater Malkawi and Mohammad 2003; Abu-Ashour and Jamrah 2008; Travis *et al.* 2010). Consequently, for safe reuse of greywater in gardens, shallow subsurface irrigation is routinely recommended to reduce risks to an 'acceptable' level (Christova-Boal *et al.* 1996). However, there is still a risk of upward movement of greywater-associated pathogens to the soil surface. Subsurface irrigation systems at a depth of 15 cm have been shown to result in movement of the wetting front to the surface (Kandelous and Šimůnek 2010). If this occurs, any pathogens present in the wastewater may come into contact with soil cover layers. Persistence or growth of pathogens in this layer would carry a potential health risk as a result of human exposure to this environment through gardening activities such as weeding, from children playing in the area or from pets transferring contamination indoors.

Little is known about the fate of these organisms in such cover layers. Organic mulches are highly effective at retaining water and moderating environmental temperatures (Chalker-Scott 2007), considered beneficial due to a reduced requirement for irrigation, an enhanced root growth and establishment of plants respectively. A higher moisture content and temperature moderation of soil also provides an environment that promotes the survival and proliferation of microbes (Chalker-Scott 2007). However, it is thought that the natural microbial populations of organic mulches may limit the growth of plant pathogens, either by direct resource competition or by the production of chemical inhibitors by the indigenous populations or the mulch itself. Little is known regarding the survival of human pathogens in soil cover layers. A study that investigated the fate of foodborne pathogens in soil covered with a variety of mulches showed persistent survival of enteric pathogens in soil under mulch, and identified straw as the mulch that facilitated the longest survival times (Micallef *et al.* 2016).

This study aimed to investigate the temporal survival of *E. coli* in three typical soil cover layers: pea straw mulch, bark mulch and coir mulch. This information will be used to make recommendations to homeowners and regulatory authorities that practice domestic greywater reuse for irrigation.

Results and discussion

No *E. coli* were detected in the control samples for any of the three mulches throughout the study, indicating that the mulches themselves were free of *E. coli* at the beginning of the investigation, and that no contamination was introduced over the course of the study.

Escherichia coli survival in bark mulch

The bark mulch showed an initial sharp decline in *E. coli* numbers for both 'greywater' and 'culture' treatments on day 3 (Fig. 1). At that sampling point, no *E. coli* were detected in the 'greywater' sample, and only 39 most probable number (MPN) per g were detected in the 'culture' treatment (Fig. 1). However, by day 7, *E. coli* numbers in samples were $6{\cdot}6 \times 10^3$ MPN per g and $5{\cdot}7 \times 10^3$ MPN per g respectively (Fig. 1). Thus, the decline on day 3 was potentially a result of *E. coli* entering a viable but nonculturable (VBNC) state under new environmental conditions, rather than a true representation of low number as a result of die-off. Bacterial cells enter a VBNC state in response to stress, such as low nutrient availability and suboptimal temperature and oxygen conditions (Oliver, 2000). In this state, cells undergo metabolic changes such as reductions in respiration rates and nutrient transport (Porter *et al.* 1995), and biochemical changes to the cell walls including increased cross-linking (Signoretto *et al.* 2002). Importantly, VBNC cells have been shown to be capable to resuscitation to a fully culturable state in response to environmental adaptation (Pinto *et al.* 2011).

Following the apparent resuscitation on day 7, *E. coli* in all bark mulch samples showed a steady decline until the end of the study, when 11 and 6 *E. coli* MPN per g were detected in the 'greywater' and 'culture' treatments respectively (Fig. 1). The *D*-values for the bark mulch were similar between the two treatments, with values calculated to be 11·28 (±0·99) for 'greywater' and 10·4 (±1·22) for 'culture'.

Bark has been reported to contain substances such as tannins, polyphenols or volatile oils (Billeaud and Zajicek 1989), which may have an antimicrobial effect on indigenous micro-organisms, as well as any pathogens introduced via exposure of the bark to greywater. These compounds may contribute to the slow decomposition rate reported for multiple bark types (Ganjegunte *et al.* 2003). High C : N

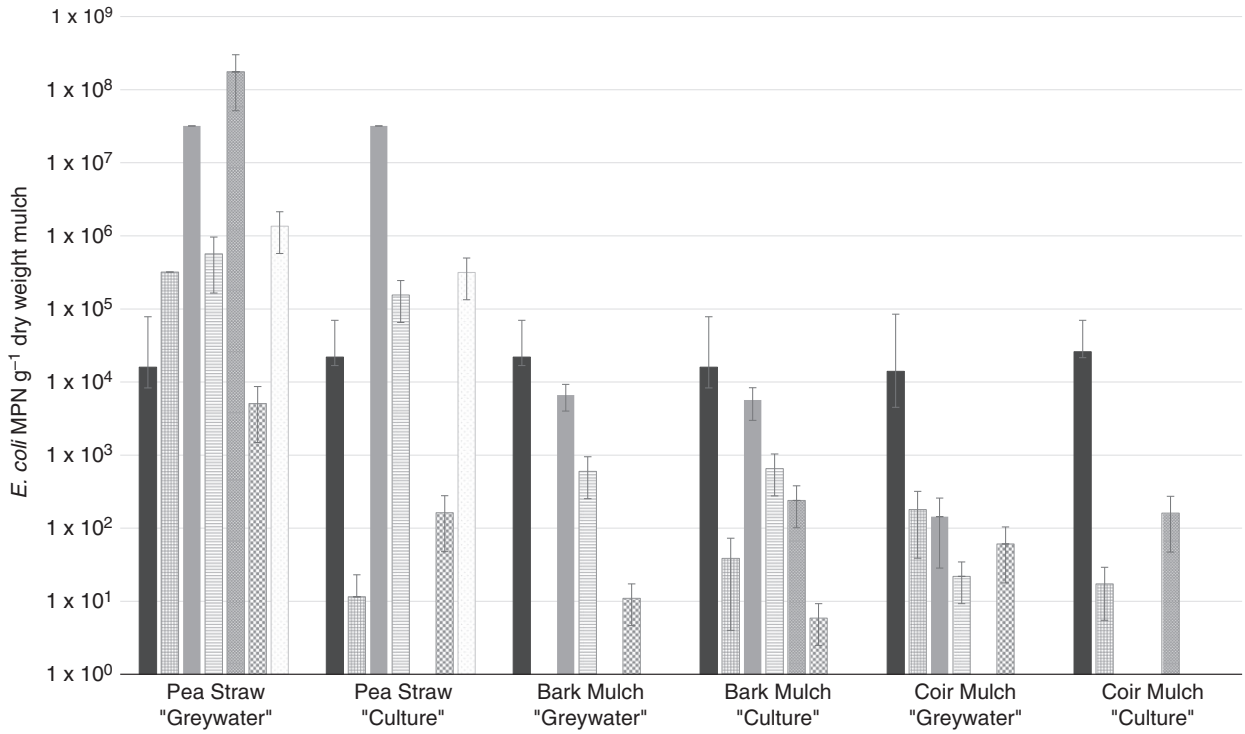

Figure 1 *Escherichia coli* most probable number (MPN) per g dry weight mulch. 'D' signifies sampling day postinoculation. Error bars denote standard error ($n = 3$). No *E. coli* were detected in any of the mulches with water only added throughout the study. (■) D0; (▦) D3; (▩) D7; (▤) D14; (▨) D21; (▧) D35 and (□) D50.

and lignin : N ratios may also contribute to slow decomposition of bark (Duryea *et al.* 1999) by providing an environment that is not conducive to microbial growth. The bark used in our study had a C : N ratio of 180 (Table 1), which is within the expected range for bark of 100–400 : 1 (Cornell Composting, 1992).

Escherichia coli survival in coir mulch

The coir mulch also showed a trend of decreasing *E. coli* levels in both treatments (Fig. 1). For the 'greywater'-treated coir mulch samples, the *E. coli* decline in viability was reasonably consistent for the duration of the trial. However, for the 'culture' treatment, the levels of *E. coli* were much more variable between sampling points and between replicates. It was difficult to obtain a representative subsample at each time point due to the nature of the mulches, particularly the large size of the chippings in the bark and coir mulches in comparison with the small sample volume. Subsamples were collected as carefully as possible to minimize this issue. Nonetheless, this variability was most obvious with the 'culture'-treated coir mulch sample, where no *E. coli* was detected on days 7 or 14, but the day 21 sample showed $1{\cdot}6 \times 10^2$ MPN per g (Fig. 1). As with the bark mulch, the *D*-values for the coir mulch were similar for both treatments, and were 2·31 (±1·98) for the 'greywater' treatment, and 0·98 (±0·12) for the 'culture' treatment. These were significantly shorter *D*-values than those observed for the bark mulch ($P < 0{\cdot}05$), indicating that this is a less favourable environment for *E. coli* survival. As with the bark, the coir mulch had a high C : N ratio of 159, with literature reporting an expected range of 75–186 : 1 (Abad *et al.* 2002).

Table 1 Analysis of the three mulches used in this study

Parameter	Pea straw	Bark	Coconut husk
Carbon (%)	44·1	48·6	49·3
Nitrogen (%)	0·94	0·27	0·31
C : N	46·9	180	159
Organic matter (%)	76·1	83·8	85·1

Escherichia coli survival in pea straw

The results of this study showed that *E. coli* was capable of not only surviving, but also proliferating, in pea straw under environmental conditions. The 'greywater' and 'culture' treatments were inoculated with $1{\cdot}6 \times 10^4$ and $2{\cdot}2 \times 10^4$ MPN per g respectively (Fig. 1). By day 7, the levels of *E. coli* in both treatments had increased to $>10^7$

MPN per g (Fig. 1). The 'greywater' treatment increased further on day 21 to $1{\cdot}8 \times 10^8$ MPN per g, the highest level of *E. coli* detected in any sample throughout the study (Fig. 1). The duration of the experiment was extended for the pea straw sample as *E. coli* were detected at $5{\cdot}1 \times 10^3$ MPN per g in the 'greywater'-treated samples on day 35 (Fig. 1). By day 50, the *E. coli* levels in the 'greywater'-treated pea straw had further increased to $1{\cdot}4 \times 10^6$ MPN per g, while the 'culture'-treated pea straw was $3{\cdot}2 \times 10^5$ MPN per g (Fig. 1). For both treatments, this was higher than the initial *E. coli* application rate (Fig. 1). Due to the lack of decline of *E. coli* during the study, *D*-values for this mulch were not calculated.

Results indicate that the pea straw was capable of supporting *E. coli* growth. Pea straw is marketed as being effective in boosting the activity of micro-organisms in the soil, and the rapid proliferation of soil microbial biomass following incorporation of crop residues has been previously documented (Jensen 1997). The pea straw contained the lowest amount of carbon and the highest amount of nitrogen of all three mulches investigated (Table 1). This resulted in a C : N ratio of 47 : 1, slightly higher than the range reported in literature for pea straw of 15–40 : 1 (Cornell Composting, 1992). The relatively higher N content of the pea straw compared to the other mulches could have reduced the competition for available N by the microbial community, resulting in favourable conditions for increased microbial proliferation (Kumar and Goh 1999). A consequence of favourable growth conditions is that it will also support the growth of pathogens that may be introduced to the mulch layer via greywater irrigation. As upward movement of greywater following subsurface irrigation is likely, the results of this study indicate that pea straw is unsuitable for use a cover layer for greywater irrigated areas of gardens.

These findings will have direct implications for regions that practice greywater irrigation as part of a water management process.

Materials and methods

Mulches

From the wide range of mulch types available, three were selected following consultation with staff at a large garden centre, based on being the most commonly purchased by customers for mulching shrubbery and border areas. The first mulch comprised a pea straw and lucerne soil conditioner that can be added to the top of soil in a garden to suppress weeds, retain moisture and add nitrogen to the soil, and thereby, boosting the activity of micro-organisms in the soil. The second mulch was composed of bark, which provides organic matter to aid in aeration and boost the activity of micro- and macro-organisms such as worms. The third was a coir mulch, composed of coconut husks. Coir mulch is recommended for decorative landscaping, retaining soil moisture and blocking weed growth.

The organic matter, total C (%), total N (%) and resulting C : N ratio were determined for each mulch (Hill Laboratories, Hamilton, New Zealand) and are shown in Table 1. Briefly, samples were oven-dried at 105°C for 24 h and ground to pass through a 2-mm screen before analysis by Dumas combustion. The moisture content was determined by overnight drying at 105°C. The water-holding capacity (WHC) of each mulch was calculated to determine the volume of each treatment solution required to reach 80% WHC.

Treatments

Each mulch was subjected to three treatments: 'greywater' was a synthetic greywater containing *E. coli*; 'culture' was a pure culture of *E. coli* made to the required concentration with triple distilled water and 'control' was a triple distilled water control.

A stock of *E. coli* solution was prepared by overnight incubation in lysogeny broth at 37°C. The broth was centrifuged at 2500 rev min^{-1} for 1 h and the pellet resuspended in Ringer's solution. The *E. coli* in this solution was enumerated by plate count on LB agar and was determined to have a concentration of 2×10^{10} CFU per ml. This was diluted further in Ringer's solution so that when 1 ml was added to the 'greywater' and 'culture' treatments applied to each of the three mulches, the final concentration of *E. coli* in each mulch was approx. 1×10^4 CFU *E. coli* per g dry weight. The initial *E. coli* concentration was selected as a reasonable dosing concentration that may result from greywater application, based on published data. Although *E. coli* levels in greywater are extremely variable, concentrations of $1{\cdot}6 \times 10^3$–$7{\cdot}4 \times 10^7$ CFU 100 per ml have been reported (Ottosan and Stenström 2003; Siggins *et al.* 2013).

The synthetic greywater was based on Jefferson *et al.* (2001), and contained bathing soap (0·005% w/v), shampoo (0·01% v/v), vegetable oil (0·001% v/v) and laundry powder (0·003% w/v). *Escherichia coli* was added to the synthetic greywater immediately prior to application to the mulch. Bulk samples of 250 g mulch were inoculated with the treatment solutions by pipette and mixed by hand for even distribution.

Sampling

A 5-g subsample was taken from each bulk mulch sample following inoculation with the treatment solution and analysed for day 0 (D0). The remaining bulk sample was divided into three replicates, which were placed in 1 l sterile glass Schott bottles, and a foam bung was inserted. All

bottles were incubated at 16°C for the remainder of the trial. A 5-g subsample of all treatments was taken on days 3, 7, 14, 21 and 35 postinoculation. In addition, the pea straw mulch was also sampled on day 50 due to an observed persistence of *E. coli*.

Escherichia coli enumeration

Enumeration of *E. coli* in each subsample was carried out using an adaption of the five-tube most probable number (MPN) method (BAM, 2002). A 5-g sample was taken from each replicate, weighed into 90 ml buffered peptone water and shaken on an orbital shaker for 1 h at 250 rev min^{-1}. Samples were serially diluted and 1 ml of each dilution was inoculated into five replicate tubes of lauryl tryptose broth with inverted glass tubes for gas collection. All tubes were incubated at 35°C for 24 h. One μl of culture from positive tubes showing gas production was transferred into EC broth + 4-methylumbelliferyl-β-D-glucuronide (MUG) and incubated at 44°C for 24 h. Negative lauryl tryptose tubes were incubated for a further 24 h and transferred to EC broth + MUG if they showed gas production. After 24 h of incubation, EC broth + MUG cultures showing gas production and blue/purple fluorescence under UV light (366 nm) were considered positive for *E. coli*.

Statistical analysis

Most probable number calculations and the corresponding confidence intervals were determined using the MPN calculator (Build 23) created by Mike Curiale (http://www.i2workout.com/mcuriale/mpn/). *D*-values are the decimal reduction times, defined as the time required at a given temperature to kill 90% of the exposed microorganisms. *D*-values were calculated from the negative reciprocal of the slope of regression lines using the linear portion of survivor curves (Horswell *et al.* 2009).

Acknowledgements

This work was supported by a New Zealand Ministry of Business, Innovation and Employment (MBIE) core funding research grant.

Conflict of Interest

No conflict of interest declared.

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