

ORIGINAL ARTICLE

Survival of *Escherichia coli* O157:H7 in wastewater from dairy lagoonsS.V. Ravva¹, C.Z. Sarreal¹, B. Duffy² and L.H. Stanker¹¹ USDA, Agriculture Research Service, Western Regional Research Center, Foodborne Contaminants Research Unit, Albany, CA, USA² Swiss Federal Research Institute for Fruit Production, Viticulture and Horticulture, Wädenswil, Switzerland**Keywords**aeration, circulated wastewater, coliforms, dairy lagoons, *E. coli* O157:H7, survival.**Correspondence**S.V. Ravva, USDA, Agricultural Research Service, Western Regional Research Center, Foodborne Contaminants Research Unit, 800 Buchanan Street, Albany, CA 94710, USA.
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Abstract**Aim:** To determine the survival of *Escherichia coli* O157:H7 in dairy wastewater from on-site holding lagoons equipped with or without circulating aerators.**Methods and Results:** Survival was monitored in dairy lagoon microcosms equipped with or without scale-size circulators. Both laboratory strains of *E. coli* O157:H7 and an isolate of *E. coli* H7 from wastewater had poor survival rates and none proliferated in water from waste lagoons with or without circulators. Furthermore, the decline of *E. coli* O157:H7 was not enhanced in those microcosms equipped with circulators. Strain variation in survival was observed in both circulated and settling waters. The decline rate of *E. coli* O157:H7 Odwalla strain increased proportionately with the inoculum load. *Escherichia coli* failed to establish itself in wastewater even after four sequential inoculations simulating continuous faecal input into the lagoon. The native aerobic bacteria survived longer with a decimal reduction time of 21.3 days *vs* either introduced or native *E. coli*, which declined rapidly with decimal reduction time of 0.5–9.4 days.**Conclusions:** *Escherichia coli* O157:H7 failed to establish and proliferate in dairy wastewater microcosms equipped with or without circulating aerators.**Significance and Impact of the study:** This study furthers our knowledge of pathogen survival in wastewater, and suggests that proper management of wastewater before its use in irrigation is essential to reduce pathogen transfer to crops.**Introduction**

An estimated 1.5 million cases of foodborne illnesses, resulting in over 17 000 hospitalizations and 600 deaths, are linked annually in the United States to infections caused by nontyphoidal *Salmonella* and enterohaemorrhagic (EHEC) *Escherichia coli* O157:H7 (Mead *et al.* 1999). *Escherichia coli* O157:H7 infections are responsible for 2001 hospitalizations and 61 deaths annually (Mead *et al.* 1999). An annual occurrence rate of eight infections per 100 000 inhabitants or higher has been reported from the United States, Japan, Scotland, Canada and United Kingdom, and the infections are five to 10 times higher in some countries of South America (Pacheco-Ferreira *et al.* 2002). Outbreaks are frequently

associated with consumption of contaminated food (meat, dairy products, fresh produce) or drinking water that was exposed to pathogen-laden animal manure or irrigation water, aerosols, and vectors tainted with manure (Beuchat and Ryu 1997; Janisiewicz *et al.* 1999; Park *et al.* 1999; Lee *et al.* 2002). Domestic livestock, particularly confined animals with long-production lives, such as dairy cows, represent an established primary reservoir for human pathogenic bacteria (Pell 1997; Himathongkham *et al.* 1999; Jones 1999). One firewall in the prevention of foodborne outbreaks is to minimize the risk of on-farm manure contamination of food products. Designing effective and sustainable intervention strategies requires an understanding of pathogen survival dynamics in farm environments.

Concentrated animal production systems that generate large volumes of manure in small areas are increasingly the norm in many regions of the world. In California, dairy operations with average herds of 900 cows are common and are designed largely as free-stall operations. California also has an intensive integrated agricultural network with large dairies located in close proximity to extensive production fields for fruits and vegetables, and other agricultural commodities. In free-stall dairies, the lanes where the cows rest and feed are most efficiently cleared of manure by regularly flushing with water that is then collected in on-site holding lagoons. Water conservation encourages the re-cycling of wastewater for lane washing and for irrigation of crops ultimately used as silage on the dairy. In terms of the life-cycle analysis of foodborne pathogenic bacteria, a pressing question is whether holding lagoons are an on-site reservoir for long-term survival or proliferation of pathogens. If so, then understanding the factors that influence pathogen competitiveness in this environment will facilitate the development of intervention strategies to break the cycle. For example, the incidence of faecal shedding of *E. coli* O157:H7 has been reported to be eight times higher in dairy herds when lanes are flushed than when alternate manure clearing methods are used (Garber *et al.* 1999). The reason for this difference is unknown, but long-term persistence (Ibekwe *et al.* 2002) and growth of pathogens (Avery *et al.* 2005) in the re-cycled water in holding lagoons would offer an explanation. Several studies have monitored on-farm shedding and prevalence of *E. coli* O157:H7 (Hancock *et al.* 1994, 1997; Garber *et al.* 1999; Laegreid *et al.* 1999; Elder *et al.* 2000), and a link has been established between high livestock densities and surface water contamination with *E. coli* O157:H7 (Johnson *et al.* 2003). However, pathogen population dynamics in dairy wastewater remains largely unknown.

Environmental survival of pathogens in solid manure and manure slurries is poorly understood, but found to be influenced by a number of factors. *Escherichia coli* O157:H7 survived for 21 months in manure from experimentally inoculated animals, when the manure was simply piled outside and exposed to fluctuating conditions, but it survived only for 47 days when stored under aerated conditions (Kudva *et al.* 1998). The rate of destruction of *E. coli* O157:H7 and *Salmonella* was observed to be temperature related during the storage of contaminated cattle and poultry manure (Himathongkham *et al.* 1999, 2000). The fastest decline was observed at increasing, but at nonlethal temperatures ranging from 4 to 37°C. *Escherichia coli* O157:H7 survived for longer than 2 months in bovine faeces at temperatures below 15°C (Bolton *et al.* 1999; Avery *et al.* 2004). A simple intervention which can result in dramatic reductions in pathogen populations in stored manure, even

at low temperatures, is aeration (Heinonen-Tanski *et al.* 1998; Kudva *et al.* 1998).

Aeration technology for manure-holding lagoons has gained popularity, because it appears to reduce odours and slime, and accelerate the decomposition of solids (Heber *et al.* 2002; Hill and Sobsey 2003). This study was aimed at determining the fate of *E. coli* O157:H7 in wastewater from dairy manure-holding lagoons with and without circulating aerators. Aerated and nonaerated microcosms were established using water from either lagoon equipped with or without circulators. Wastewater was collected from the lagoons over a period of 18 months to examine the influence of seasonal fluctuations in factors that may affect pathogen survival (e.g. microbial communities and chemical changes). Variation in survival among different pathogen strains and the impact of pathogen loading (e.g. re-introduction by re-cycling wastewater for lane flushing) were examined.

Materials and methods

Manure wastewater

Flush water was collected from aerated and nonaerated manure lagoons from two medium-sized (c. 800 milking head) dairies (Oakdale, CA, USA). These dairies were chosen for their similarities in lagoon size, water characteristics, and dairy management practices. Both the dairies were located within a kilometre of each other, were exposed to the same weather, and the cows were fed similar diets formulated by animal nutritionists. Both the lagoons had approximately the same holding capacity (c. 95×10^6 l) with similar loading rates. The only noticeable difference between these two dairies is the use of three vortex CirCulatorsTM (Circul8 Systems, Reardon, WA, USA; Rumburg *et al.* 2004) by dairy A in its lagoon. Dairy A separates manure solids by holding the alley flush water for <24 h in a temporary settling pit (c. 1×10^6 l) to allow the manure solids to settle. Water from the settling pit is then pumped into the lagoon equipped with circulators. The circulators continuously mix the water in the lagoon bringing water from the bottom to the surface. Dairy A uses the water from its circulated lagoon for flushing the free-stall lanes and to irrigate and fertilize pastures. Dairy B did not use circulators and also re-cycled its water from the stationary lagoons to flush the lanes.

Isolation of *Escherichia coli* O157:H7 from manure and wastewater

Approximately 100 samples from dairies A and B were collected during summer and winter months on six

occasions over a 2-year period. Samples included pooled fresh manure, wastewater from circulated and settling lagoons, and dry manure from on-site manure piles. One millilitre or 1 g of samples were inoculated into 9 ml of GN broth (Difco, Detroit, MI, USA) supplemented with vancomycin, cefixime, and cefsulodin (Elder *et al.* 2000) to enrich *E. coli* O157:H7. Anti-*E. coli* O157 Dynabeads® were used following the manufacturer's instructions (DynaL Biotech Inc., Lake Success, NY, USA) for low-level detection from wastewaters and enrichments. One hundred-microlitre portions of serial dilutions of wastewaters and enrichments were plated on sorbitol MacConkey agar supplemented with cefixime and tellurite. Enrichments and plated media were incubated at 37°C for 24 h.

Wastewater microcosms to monitor the fate of *Escherichia coli* O157:H7

Freshly collected wastewater, acclimated overnight at room temperature, was used to prepare the microcosms in order to monitor the fate of inoculated pathogens. All pathogen fate studies were conducted at inocula of 10^5 – 10^7 CFU ml⁻¹. The tests were conducted in either 1-l microcosms, magnetic culture vessel fitted with stir bar suspended from the closure (Nalgene), or in large cylindrical jars (6.6 l, 152 × 457 mm, Kimax) aerated with miniaturized overhead re-circulating mixers (Circul8 Systems). Aeration was maintained by re-circulating the water column in a gentle vortex motion without turbulence. The mixing process, as optimized by the manufacturer, closely simulates the re-circulation observed in wastewater lagoons equipped with commercial circulators (Circul8 Systems). Samples were collected at the circulating zone of the mini re-circulators, which is approximately 15 cm below the surface. Nonaerated microcosms were sampled at the same depth. Microcosms prepared using wastewater from the lagoon equipped with circulators were incubated under mixing, while water from the

lagoon without circulators was used to produce nonaerated microcosms. The microcosms were incubated at $23 \pm 2^\circ\text{C}$. The microcosms were sampled for 6 weeks or until the introduced organisms could no longer be detected on two consecutive samplings. Zero time samplings were done 1 h after inoculation to the wastewaters. The die off of the organism during acclimation is ignored in survival rate determinations. The temperature of the un-inoculated microcosms was monitored continuously with an immersion probe and a Dickson FT121 electronic recorder (Dickson, Addison, IL, USA). Dissolved oxygen was measured with an YSI 5100 oxygen metre equipped with a 5010 self-stirring BOD probe (YSI, Inc., Yellow Springs, OH, USA), and pH was monitored with an Accumet AP63 pH metre (Accumet-Fisher, Hampton, NH, USA). Chemical analysis of wastewaters from both dairies was performed (A & L Western Agricultural Labs. Inc., Modesto, CA, USA).

Enumeration of pathogens and native bacteria

Both outbreak and naturally occurring strains of *E. coli* O157:H7 (Table 1) used to inoculate the microcosms were selected for spontaneous rifampicin and nalidixic acid resistance. Survival was monitored by plating 100- μ l portions of serial dilutions of wastewater in phosphate buffered saline (0.01 mol l⁻¹, pH 7.4) on Luria-Bertani (LB) agar supplemented with 100 μ g ml⁻¹ of rifampicin and 50 μ g ml⁻¹ each of nalidixic acid and cycloheximide (LB-RNC agar). The presence of additional nalidixic acid resistance was necessary for differentiating the native rifampicin-resistant (*rif^R*) colonies from the test strains used. Cycloheximide was used in the plating media to control fungi. The detection of pathogens from undiluted manure water was improved by plating triplicate 100- μ l samples and pooling the counts. The detection of low-level and sublethally injured cells was accomplished by 24-h pre-enrichment of 1 ml of wastewater in 9 ml of LB

Table 1 Bacteria used in wastewater survival tests

| Organisms* | Strain no. | Source | Details |
|---------------------------------|------------|-------------------------------|--|
| <i>Escherichia coli</i> O157:H7 | MM100 | Odwalla orange juice outbreak | FDA strain no. SEA13B88 |
| <i>E. coli</i> O157:H7 | MM149 | Dairy manure | Isolated 8/93, NW Oregon |
| <i>E. coli</i> O157:H7 | MM202 | Dairy manure | Isolated 8/94, West Washington |
| <i>E. coli</i> H7† | MM158 | 4-week-old calf | California veal farm, VMTRC#8051-B (Veterinary Medical Teaching and Research Center, Tulare, CA, USA). |
| <i>E. coli</i> H7†‡ | MM260 | Circulated lagoon of dairy A | Isolated during this study |

*Organisms were sequentially screened for rifampicin (100 μ g ml⁻¹) and nalidixic acid (50 μ g ml⁻¹) resistance. Colonies were selected on Luria-Bertani (LB) agar supplemented with incremental amounts of antibiotics (10–25 μ g ml⁻¹) in each screening cycle. Wild-type strains were from the Produce Safety and Microbiology Unit, USDA, ARS, Albany, CA, USA.

†Serotyped by ELISA. Not reactive with O126, O111, and O157 antibodies.

‡Identified by 16S rDNA sequencing (Microbe Inotech Labs., St Louis, MO, USA).

broth supplemented with 100- $\mu\text{g ml}^{-1}$ rifampicin (LB-R broth). Tenfold serial dilutions of enrichments were plated on LB-RNC agar for qualitative detection of low level and stressed cells that could not be detected by direct plating of wastewater dilutions. The populations of native coliform and aerobic bacteria were monitored by analysing 1-ml dilutions of samples on petrifilm *E. coli*/coliform and aerobic count plates (3M Microbiology Products, St Paul, MN, USA). Except for aerobic count plates that were incubated at 25°C, the enrichments and enumeration plates were incubated at 37°C. *Escherichia coli* O157:H7 from un-inoculated wastewater were monitored as described previously.

Fate of *Escherichia coli* O157:H7 in wastewaters at different inoculum levels

The influence of inoculum load on survival was monitored using *E. coli* O157:H7 strain MM100. Four separate 6-l circulated wastewater microcosms were established using dairy A circulated water. Inoculum levels ranged from 7×10^3 to 1×10^7 CFU ml^{-1} . Likewise, wastewater from the temporary settling pit (from dairy A) was used to prepare four 6-l noncirculated microcosms, and these were inoculated as the circulated microcosms. The microcosms were monitored for pathogen decline at 0, 1, 2, 5, 8, 14, and 21-day intervals. To control the risk of pathogen spread through aerosols, these large open vessels with or without overhead circulators were incubated inside level 2 biological safety hoods. To avoid cross-contamination through aerosols, the noncirculated microcosms were incubated in a separate hood. The organisms were also monitored at different depths (2, 8, 20, and 40 cm) of the 41-cm water column to determine the influence of oxygen gradient on their survival. Dissolved oxygen was measured from the same sampling depths.

Fate of *Escherichia coli* O157:H7 in wastewaters from circulated and settling lagoons

The survival of *E. coli* strains MM149 and MM158 was evaluated between February 2002 and April 2003 (eight trials) in freshly collected circulated and noncirculated wastewaters from dairy A. Survival of the same two strains was also evaluated in triplicate microcosms established with circulated wastewater from dairy A, and compared with noncirculated water from dairy B during September 2002–April 2003 (four trials). One-litre microcosms were established in these comparisons.

In addition to the aforementioned comparisons, the survival of five different strains of *E. coli* (Table 1) was also monitored in wastewaters collected 15 times from both dairies during a period of 17 months to determine

strain variations and seasonal influence on pathogen fate. About two to three replicated microcosms were used in these comparisons. The microcosms were sampled for 6 weeks or until the introduced organisms could no longer be detected on two consecutive samplings. Native coliforms and aerobic bacteria were also monitored.

Acclimation of *Escherichia coli* O157:H7 in wastewaters

The continuous shedding of pathogens and their potential acclimation in re-cycled wastewater used for lane washings was simulated by repeated inoculations with strain MM158 into 6-l microcosms. The organism was introduced at 3×10^5 CFU ml^{-1} into microcosms that were established using water from circulated and settling lagoons on dairy A. The microcosms were re-inoculated at the same level on days 17, 28, and 36. This was accomplished by the addition of 8-ml overnight growth of MM158 in LB-RN broth with an OD adjusted to 0.35 at 600 nm. The populations of MM158 in microcosms were monitored before and after inoculations and at various intervals by plating the dilutions of wastewater in phosphate-buffered saline on LB-RNC agar.

Fate of native bacteria from wastewater

Native bacteria in wastewaters from circulated (dairy A) and settling (dairy B) lagoons were acclimated to tolerate 100 $\mu\text{g ml}^{-1}$ of rifampicin by incubating 1 l of each water treated with the antibiotic for 24 h at 23°C. The organisms were concentrated at 4000 g for 20 min at 4°C, and reconstituted in 100 ml of the source wastewater without rifampicin. Ten-millilitre portions of each concentrate containing *rif^R* organisms were inoculated into triplicate 250-ml Erlenmeyer flasks, each containing 90 ml of water from circulated and noncirculated lagoons from dairies A and B, respectively, and incubated for 67 days. The treatments of circulated water were incubated on a gyratory shaker operated at 120 rev min^{-1} . The *rif^R* bacteria in microcosms were monitored at various intervals by plating 100 μl of serial dilutions of wastewater on LB agar supplemented with 100 $\mu\text{g ml}^{-1}$ of rifampicin and 50 $\mu\text{g ml}^{-1}$ of cycloheximide.

Statistical analysis

The pathogen decline numbers from each microcosm were transformed logarithmically and the days required for one log decline (*D*-value) were calculated from linear regression of log cell number decline over time. Analysis of variance (ANOVA) tests (SigmaStat 3.0; SPSS Inc., Chicago, IL, USA) were performed to determine if

the circulation of wastewater, the dates of water collection, and the strains of *E. coli* had any influence on the survival of organisms in wastewater. Specifically, two-way ANOVA tests were performed on *D*-value data with circulation status of wastewater and strains as treatment factors. If differences in least square mean *D*-values between the strains were not significant, then two-way ANOVA was performed to determine the seasonal influence on survival by analysing circulation status and wastewater collection dates as treatment factors. The strain differences were ignored in these tests. ANOVA tests were performed at $P > 0.05$ for normality and equal variance tests and the test of significance was set at $P = 0.05$.

Results

Detection of *Escherichia coli* O157:H7 in wastewaters and monitoring its survival at different inoculum levels

Escherichia coli O157:H7 was not detected in manure and wastewater samples from dairies A and B. Efforts to isolate *E. coli* O157:H7 by direct plating of dilutions of manure and wastewater in selective isolation media or by immuno-magnetic separation followed by plating on selective media were unsuccessful. As the pathogen was not detected in wastewaters, the fate of naturally occurring *E. coli* O157:H7 could not be monitored. *Escherichia coli* O157:H7 Odwalla strain (MM100), inoculated at different levels (7×10^3 – 1×10^7 CFU ml⁻¹), declined rapidly in 6-l microcosms established with water from either the circulated lagoon or the settling pit from dairy A (Fig. 1). The rate of decline of the Odwalla strain was identical in both the microcosms, and in general, the rate of decline of the introduced pathogen increased proportionately ($r^2 = 0.997$) as the inoculum increased from 10^3 – 10^7 CFU ml⁻¹ in both the aerated and nonaerated microcosms. *Escherichia coli* O157:H7 Odwalla declined to undetectable levels within 14 days regardless of the inoculum level or aeration status. An identical population decline was observed in those samples taken at four depths (2–40 cm) from both circulated and noncirculated microcosms (data not shown).

We observed no difference in decline rate for *E. coli* O157:H7 strain MM149 (*D*-value = 3.0 ± 0.5 days) in 1- and 6-l microcosms established using circulated wastewater from dairy A. Thus, 1-l microcosms were used in all the subsequent comparisons of the survival of pathogen in wastewaters. No dissolved oxygen was detected in any of the samples from both the circulated and noncirculated microcosms. No change in pH was observed in wastewater microcosms during the incubations.

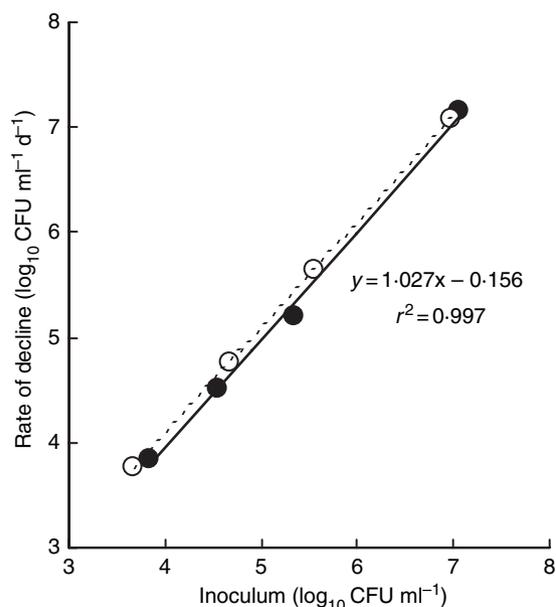


Figure 1 Fate of *Escherichia coli* O157:H7 (MM100) at different inoculum levels in 6-l manure water microcosms from circulated lagoon (●) and settling pit (○) from dairy A. Rates of decline were from 24-h incubations. Regression equation for decline in aerated water is shown.

Detection of low level and stressed cells of *Escherichia coli* O157:H7 in wastewaters

In an attempt to detect low levels and stressed cells, the survival of three strains of *E. coli* O157:H7 (MM100, MM149, and MM202) in circulated wastewater from dairy A was monitored before and after 24-h pre-enrichments in LB-R broth followed by plating on LB-RNC agar. Pre-enrichments were designed to qualitatively detect low levels of cells. Consequently, the enriched counts were not used in *D*-value determinations. All three strains behaved similarly and declined rapidly with *D*-values of 1–2 days as measured by direct plating of wastewater dilutions. In an example shown in Fig. 2, strain MM100 declined rapidly to undetectable levels in <6 days by direct plating, but was detected at high population levels for up to 8 days as a result of pre-enrichments. A high proportion of cells were detected after pre-enrichments, while they were not detected by direct plating of wastewater dilutions. Furthermore, the other two strains could not be detected after 8 days by direct plating and after 2 weeks with pre-enrichments.

Survival of *Escherichia coli* in wastewater from lagoons without or with circulators

The influence of continuous circulation of the lagoons on the survival of *E. coli* strains MM158 and MM149 in

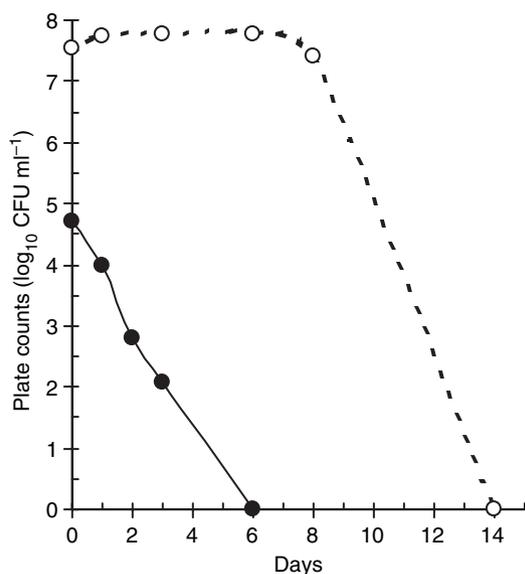


Figure 2 Detection of stressed cells of *Escherichia coli* O157:H7 strain MM100 in circulated wastewater from dairy A. The organism was enumerated on Luria-Bertani-rifampicin nalidixic acid cycloheximide (LB-RNC) agar plates by plating serial dilutions of wastewater (●) and the 24-h pre-enrichments of wastewater in LB-R broth (○).

wastewaters collected from dairies A and B was monitored (Table 2). The inoculated organisms failed to grow in waters from either dairy and they declined rapidly in both the circulated and unmixed microcosms. The data also indicate that circulation of the lagoon did not accelerate the decline of pathogenic *E. coli*. No significant difference in the mean *D*-values for *E. coli* strains was observed for tests in circulated water from dairy A compared with settling waters from either dairy A ($P = 0.87$) or dairy B ($P = 0.15$). An example of identical population decline of strain MM149 ($D = 6.4 \pm 0.5$ days) from both circulated and noncirculated wastewaters is shown in Fig. 3. The native coliforms were observed to decline rapidly with decimal reductions comparable ($D = 7.6$ days)

with those observed with the introduced *E. coli* strains. In contrast, native aerobic bacteria persisted ($D = 27.7$ days) in these microcosms using both circulated and settling water (Fig. 3). Native coliforms were generally not detected after 2 weeks. Dissolved oxygen was not detected at any instance during the incubations in either the circulated or unmixed wastewater microcosms.

Seasonal influence on survival of introduced *Escherichia coli* in wastewaters

Seasonal influence on survival of two *E. coli* strains (MM149 and MM158) was measured in microcosms prepared with circulated water from dairy A and noncirculated water from dairy B. A significant seasonal influence (four samplings, $P = 0.004$; two aerations, $P = 0.084$; two-way ANOVA) on reduction of both strains was observed only in waters collected during November 2002 (mean $D = 5.1$ days) as compared with water collected during April 2003 (mean $D = 2.5$ days). Pathogen decline was not significantly different in wastewaters collected during the months of September 2002 (mean $D = 3.0$ days), December 2002 (mean $D = 3.9$ days), and April 2003. Both strains declined similarly ($P = 0.736$) from wastewaters at all sampling intervals. An example of the seasonality on the survival of MM149 in circulated water from dairy A is shown in Fig. 4. The *D*-value appeared to be inversely related to the average monthly temperature ($r = -0.6$) of Oakdale, CA (<http://www.city-data.com/city/East-Oakdale-California.html>). As the temperature declined from 23 to 12°C during September to November, the *D*-value increased from 1.5 to 5.9 days. Tests using samples collected during November to December also resulted in longer survival of three of the five *E. coli* strains (Table 3) compared. In addition, *D*-values from individual microcosm tests conducted during several months from both dairies ranged between 0.5 and 9.4 days (Table 3). However, the survival of *E. coli* could

Table 2 Influence of circulating aeration on the survival of *Escherichia coli* in manure wastewaters from two dairies

| Wastewater comparisons* | No. of trials | Replicates | Mean <i>D</i> -value, d† | | | | | |
|-----------------------------|---------------|------------|--------------------------|----------|-----------------|---------|-------|-----------------|
| | | | Circulation | | | Strains | | |
| | | | Circulated | Settling | <i>P</i> -value | MM149 | MM158 | <i>P</i> -value |
| Circulated A and settling A | 8 | 2 | 3.1 | 3.0 | 0.87 | 2.5 | 3.1 | 0.18 |
| Circulated A and settling B | 4 | 3 | 3.2 | 4.2 | 0.15 | 3.6 | 3.8 | 0.74 |

*Tests in 1-l microcosms. Survival in wastewaters from circulated lagoon of dairy A was compared with temporary settling lagoon of dairy A and permanent settling lagoon of dairy B. Microcosms of circulated wastewater were continuously mixed during the incubations, while the settling water microcosms were not.

†Least square mean *D*-values are from two-way ANOVA with water source and strains as treatment factors. The seasonal influence on survival was evaluated separately.

Figure 3 Similarity in the decline of *Escherichia coli* O157:H7 strain MM149 in wastewater from circulated lagoon in dairy A (\blacktriangle , solid line) and settling lagoon from dairy B (\triangle , broken line). Total aerobic bacterial (\bullet) and coliform (\blacklozenge) plate counts of wastewater from circulated lagoon are shown. Aerobic bacterial and coliform counts from settling lagoon water from dairy B are similar to the counts from the circulated lagoon of dairy A and are not shown. Error bars represent SD from the mean of three replicates.

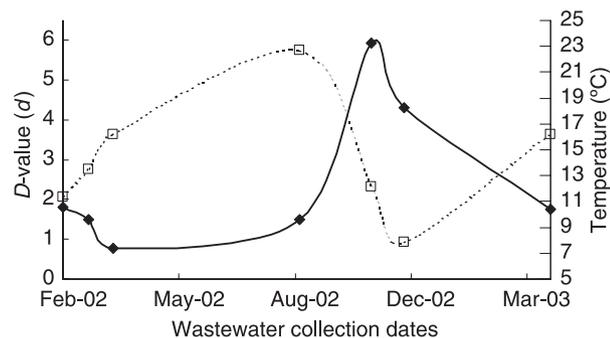
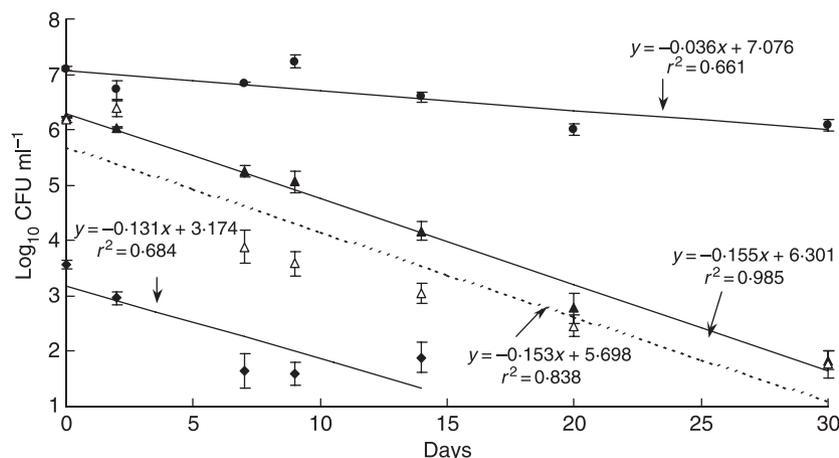


Figure 4 Seasonal influence of temperature on the survival of *Escherichia coli* O157:H7 strain MM149 in circulated manure wastewater from dairy A. Average monthly temperatures (\square) were plotted against decimal reduction values (\blacklozenge) for MM149.

not be correlated with any nutrients, organic matter, biological oxygen demand (BOD), or chemical oxygen demand (COD) of wastewaters collected at different sampling intervals (data not shown). Representative data of chemical analysis of wastewaters from three sampling intervals is shown in Table 4.

Strain variations in survival

In addition to their rapid decline, strain variation was observed in the survival of *E. coli* (Fig. 5) in microcosms prepared with water collected from both circulated and settling lagoons from dairy A. Similar results were obtained in tests conducted using the water collected from the same dairy at one or more intervals (data not shown). Mean *D*-values for strains tested in wastewaters from dairy A ranged from 0.7 to 6.1 days (Fig. 5). Compared with these *D*-values, the strains persisted in wastewaters between 3 and >38 days. Significant differences were observed between the strains ($P = 0.009$) and the

Table 3 Range of *D*-values obtained for *Escherichia coli* strains from all microcosm tests using wastewaters from dairies A and B

| Strain# | <i>D</i> -value* (lowest/highest) | Wastewater source | Water collection date |
|---------|-----------------------------------|-------------------|-----------------------|
| MM100 | 0.5 | Settling A | February 2002 |
| | 7.0 | Circulated A | December 2001 |
| MM149 | 0.6 | Settling A | February 2002 |
| | 6.8 | Circulated A | November 2002 |
| MM202 | 0.5 | Settling A | March 2002 |
| | 3.0 | Circulated A | March 2002 |
| MM158 | 1.9 | Settling A | February 2002 |
| | 9.4 | Settling B | November 2002 |
| MM260 | 4.1 | Settling A | September 2002 |
| | 8.8 | Circulated A | July 2002 |

*Lowest to highest *D*-values are from 15 different tests conducted during December 2001–April 2003. Wastewaters, from which the extreme *D*-values were obtained and the corresponding water collection dates are shown.

mixing status ($P = 0.022$; mean *D*-value for circulated = 3.7 days, *D*-value for settling = 2.5 days) of water from dairy A. However, there was no statistically significant interaction between the strains and the circulation status ($P = 0.359$). An *E. coli* H7 isolate (MM260) from the circulated lagoon survived longer in both circulated and settling waters (Fig. 5). Nevertheless, a different *E. coli* H7 isolate (MM158), survived longest in settling water from dairy B collected during November ($D = 9.4$ days; Table 3). This organism was detected at 4×10^2 CFU ml⁻¹ even after 38 days in the microcosms.

Acclimation of *Escherichia coli* O157:H7 in wastewater

In a study that simulated pathogen loading of lagoons by re-introduction of re-cycled wastewater with manure, MM158 failed to acclimate during four inoculations to water from dairy A incubated for 45 days (Fig. 6).

Table 4 Chemical characteristics of manure wastewater from two dairies

| | September 2002 | | | November 2002 | | | January 2003 | | |
|------------------------------|----------------|----------------|-------------------|---------------|----------------|-------------------|--------------|----------------|-------------------|
| | Circulated A | Settling pit A | Settling lagoon B | Circulated A | Settling pit A | Settling lagoon B | Circulated A | Settling pit A | Settling lagoon B |
| N | 268 | 265 | 512 | 351 | 519 | 133 | 1284 | 1476 | 1022 |
| P | 89 | 86 | 53 | 122 | 107 | 180 | 67 | 105 | 107 |
| K | 420 | 413 | 363 | 527 | 559 | 583 | 695 | 813 | 811 |
| S | 45 | 31 | 7 | 79 | 15 | 103 | 69 | 90 | 36 |
| Mg | 92 | 113 | 102 | 142 | 118 | 170 | 129 | 158 | 120 |
| Ca | 94 | 174 | 140 | 234 | 358 | 314 | 255 | 332 | 182 |
| Na | 213 | 202 | 151 | 283 | 151 | 307 | 342 | 394 | 185 |
| Fe | 57 | 26 | 4.8 | 9.9 | 8.5 | 40 | 9.1 | 8.8 | 11 |
| Al | 25 | 13 | 1.7 | 5.0 | 6.0 | 19 | 5.6 | 7.9 | 9.8 |
| Mn | 0.4 | 2.1 | 0.6 | 1.6 | 1.0 | 2.9 | 1.5 | 2.3 | 1.3 |
| Cu | <0.1 | 0.7 | <0.1 | 0.3 | 0.4 | 0.1 | 0.5 | 0.6 | 0.5 |
| Zn | 0.1 | 2.6 | <0.1 | 1.1 | 0.8 | 2.9 | 1.8 | 2.2 | 1.4 |
| NO ₃ ⁻ | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | 0.6 | <0.5 |
| NH ₄ ⁺ | 230 | 232 | 270 | 247 | 293 | 259 | 396 | 642 | 332 |
| EC | 3.6 | 4.2 | 4.9 | 5.4 | 5.9 | 4.9 | 8.0 | 9.3 | 5.6 |
| OM | 1300 | 4200 | 1800 | 1590 | 2989 | 1298 | 3873 | 5701 | 2298 |
| BOD | 561 | 579 | 578 | 514 | 792 | 183 | 2163 | 2410 | 927 |
| COD | 1947 | 2109 | 1825 | 3193 | 6798 | 1916 | 1980 | 2500 | 460 |
| TSS | 400 | 545 | 230 | 1770 | 4330 | 1180 | 1867 | 2765 | 393 |

Values are in $\mu\text{g ml}^{-1}$ except for electrical conductivity (EC), which is expressed as mS cm^{-1} ; OM, organic matter; BOD, biological oxygen demand; COD, chemical oxygen demand; TSS, total suspended solids.

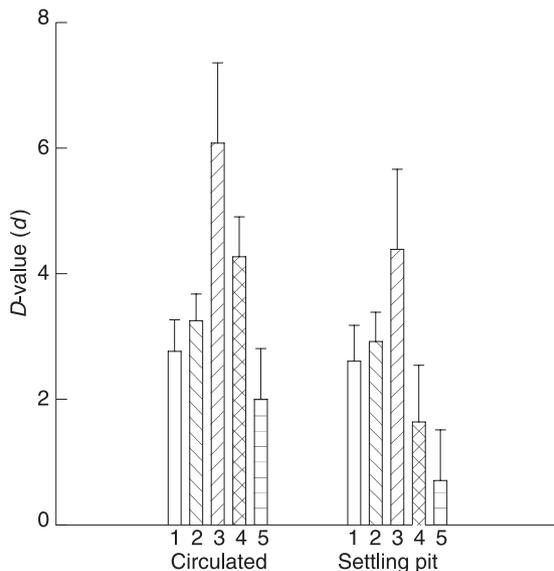


Figure 5 Strain variations in the survival of *Escherichia coli* in circulated and settling pit wastewaters from dairy A. The *Escherichia coli* strains compared were: (1) MM149; (2) MM158; (3) MM260; (4) MM100, and (5) MM202.

Although the populations declined slowly ($D = 7.9$ days) during the first inoculation, they declined rapidly during the subsequent re-inoculations. D -value after the fourth

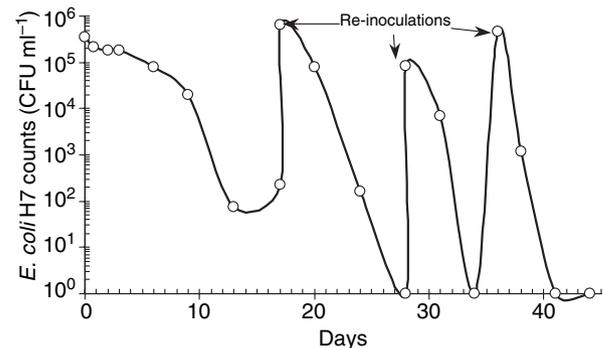


Figure 6 Acclimation of *Escherichia coli* H7 strain MM158 during four inoculations to circulated manure lagoon water from dairy A.

inoculation was 0.9 days. MM158 also failed to acclimate in wastewater from settling pit, and declined at the same rate as in wastewater from the circulated lagoon.

Survival of native bacteria from wastewaters

Nutrient limitation in laboratory microcosms could be a reason for the inability of introduced pathogens to survive and proliferate in wastewater. Microcosms were validated to see if the nutrients in wastewater can support growth of a tenfold increase in native organisms during a 67-day trial. In addition, the native *rif^R* bacteria from

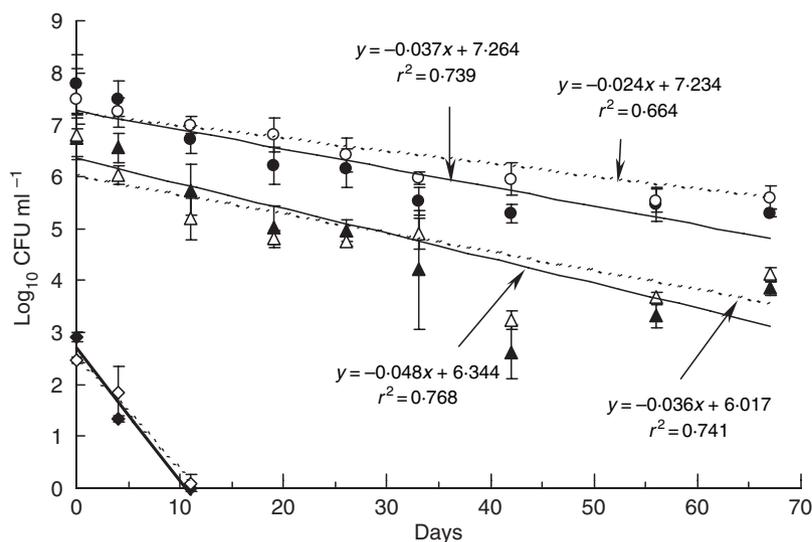


Figure 7 Fate of native rifampicin-resistant (*rif^R*) bacteria (▲, △), total aerobic bacteria (●, ○), and coliforms (◆, ◇) in circulated manure water from dairy A (solid lines; ●, ▲, ◆) and settling lagoon water from dairy B (broken lines; ○, △, ◇). The field circulated wastewater was mixed during incubations. Error bars represent SD from the mean of three replicates.

wastewaters were monitored as surrogates for introduced *rif^R* pathogens. Bacterial colonies of different colours and shapes were enumerated on LB agar, supplemented with rifampicin, and these *rif^R* bacteria represented 7×10^5 – 8×10^5 CFU ml⁻¹ in both circulated and settling waters. Surprisingly, *rif^R* bacteria were also present in high numbers (2×10^5 – 3×10^5 CFU ml⁻¹) in untreated wastewaters and they represent ~1–2% of total aerobic bacterial counts ($1.4 \pm 1.1 \times 10^7$ CFU ml⁻¹). Native *rif^R* bacteria declined slowly from both circulated and settling wastewaters (Fig. 7). *D*-value for *rif^R* bacteria from both waters was 21.3 ± 1.2 days. Total aerobic bacterial populations also declined at the same rate as the *rif^R* bacteria. However, the native coliforms declined from 10^3 CFU ml⁻¹ to undetectable levels in less than 10 days (Fig. 7). *D*-value for coliforms from both waters was 3.8 ± 0.8 days. Coliform and aerobic bacterial counts were similar in waters from both dairies.

Discussion

Contamination of dairy, meat, fruit, and vegetable products with foodborne pathogenic bacteria can be related to direct or indirect exposure to pathogen-laden animal wastes (Pell 1997). Finding the on-farm site(s) of pathogen survival and proliferation will facilitate the development of more effective and targeted intervention strategies to minimize food safety risks while still making greater use of manure as a valuable agricultural resource. Pathogen survival in solid manure and manure slurries has been studied, but little is known of the fate in manure lagoons. We were unable to detect *E. coli* O157:H7 in a survey of a wide range of samples that include lagoon water, from two California dairies by using plating,

enrichment and immuno-magnetic bead-enhanced isolation methods, sensitive for sampling solid manure and water (Hancock *et al.* 1994, 1997; Elder *et al.* 2000; Johnson *et al.* 2003). The failure to detect *E. coli* O157:H7 is not surprising, as Vernozy-Rozand *et al.* (2002) could find only one O157:H7 strain from 752 samples of manure slurries, manure, compost, and municipal sewage in France. On the contrary, *E. coli* O157:H7 was detected in high numbers throughout the year from dairy wastewater wetlands (Ibekwe *et al.* 2002). As pathogens were not detected in wastewaters, we studied the pathogen fate in laboratory models using wastewater collected from Central California dairy lagoons. We found that pathogens were noncompetitive and unable to establish when introduced into the wastewater. We also observed a proportional decline of *E. coli* O157:H7 Odwalla populations when the inoculum was reduced from 10^7 to 10^4 CFU ml⁻¹. Thus, subsequent studies on survival were made at 10^5 – 10^7 CFU ml⁻¹. We were surprised to find a high-resident population (10^5 CFU ml⁻¹) of bacteria resistant to rifampicin in the wastewater. This did not interfere with the detection of introduced strains that were marked with resistance to both rifampicin and nalidixic acid, but it does raise questions of cross-resistance development in populations of bacteria in wastewater. However, natural resistance to rifampicin appears to be a common trait in pathogens (Stock and Wiedemann 1999, 2000, 2002) likely to be present in animal wastes (Pell 1997). We also found that *E. coli* H7 failed to acclimate and declined following repeated loading of the microcosms with pathogens mimicking the repeated loading that occurs on a dairy when wastewater is re-cycled for lane flushing. A more rapid decline was observed under the conditions of re-inoculations.

Introduced *E. coli* O157:H7 and *E. coli* H7 declined by 90% within 1–10 days in all trials, and in general, disappeared in less than 4 weeks. Similar decimal reduction times of 3–15 days (Himathongkham *et al.* 1999) have been reported for *E. coli* O157:H7 in manure slurries. Temperature plays a role in pathogen destruction, with decline being generally greater at higher temperatures (Heinonen-Tanski *et al.* 1998; Himathongkham *et al.* 2000; Jiang *et al.* 2002, 2003; Guan and Holley 2003). Seasonal temperature fluctuations have an indirect influence on pathogen decline. This suggests that decline should be even greater on farms in California, where the seasonal temperatures often exceed 23°C, which is the temperature used in our laboratory trials. We observed a higher decimal reduction time for *E. coli* O157:H7 in tests with wastewaters collected during the winter as compared with the summer months. The observed dramatic increase in *D*-value for MM149 from September to November, as the temperature dropped from 23 to 12°C clearly demonstrates the seasonal influence on the survival of pathogens in wastewater.

Of the five *E. coli* strains evaluated, only one had consistently higher *D*-values, suggesting that genetic differences between strains can predispose the bacteria to respond differently to the chemical and biological components of the lagoon water. Strain-dependent variability has been reported for *E. coli* survival in soil (Topp *et al.* 2003) and seawater environments (Rigsbee *et al.* 1997). Strains that survive longer in wastewater may have an increased probability of re-infecting the animals. Thus, determining the genetic, environmental, and biological factors that influence the proliferation of selective strains of pathogens is critical in understanding and preventing pathogen outbreaks linked to manure.

The inability of introduced pathogens to establish and proliferate in wastewater may be due to competition from indigenous micro-organisms. As the community interactions are complex, we only monitored the influence of introduced pathogens on populations of aerobic bacteria and coliforms. Aerobic bacteria were maintained at high levels in the presence or absence of introduced pathogens. This is not surprising, because they were native to the wastewater, and probably had an advantage over introduced pathogens. Wastewater from both dairies had high levels of nutrients adequate to support the native populations. As a result, the *rif^R* bacterial populations in our microcosms declined slowly with a 90% reduction in 21 days as compared with 0.5–9 days for *E. coli* O157:H7 or H7 strains. The similarity in the survival of native *rif^R* bacteria and the total aerobic bacterial populations from wastewater was unexpected and raises concerns about antibiotic resistance in dairy environments. In contrast, native coli-

forms were observed to decline as rapidly as the introduced pathogens.

The rapid decline of *E. coli* strains in the absence of dissolved oxygen in microcosms suggests that other factors may be more significant for their survival in manure lagoons. It is surprising that *E. coli* failed to survive in oxygen-deficient wastewaters that are likely to be conducive for the growth of facultative anaerobes. Similar to the microcosms, dissolved oxygen was also not detected in lagoons installed with CirCulators™ (Rumburg *et al.* 2004). Other factors that may play a role in pathogen decline, such as seasonal changes in the chemistry and biology of wastewater, need to be investigated. The release of ammonia and carbonate at high pH is known to reduce *E. coli* O157:H7 (Himathongkham *et al.* 2000; Russell and Jarvis 2001; Park and Diez-Gonzalez 2003). Likewise, volatile fatty acid release, as pH declines under anaerobic conditions, reduces *E. coli* O157:H7 (Harris *et al.* 2001). As *E. coli* O157:H7 declined with *D*-values less than 9.4 days in water from lagoons, holding the contaminated water (Vernozy-Rozand *et al.* 2002) for sufficient cycles of reduction should yield higher quality water for crop irrigations. Such reduction in naturally occurring *E. coli* O157:H7 was achieved by holding the wastewater in constructed wetlands (Ibekwe *et al.* 2002).

We found that pathogenic and nonpathogenic *E. coli* added to our microcosms were unable to proliferate in wastewater from lagoons. However, even low numbers of pathogens may pose an epidemiological risk if they escape into environments that favour their re-growth. The deadly Walkerton, Canada outbreak of *E. coli* O157:H7 was caused by contamination of drinking water with low levels of pathogen that established and increased in biofilms (Holme 2003). We have found that in more complex water environments, introduced pathogens re-grow only in the presence of an added carbon source (Duffy *et al.* 2004). Food safety risks may occur at numerous points as a result of pathogen re-growth. We have found that pathogens can survive on crops (Duffy 2003), and are examining if pathogen re-growth is a source of infection. The recurrence of outbreaks (DeWaal *et al.* 2005) resulting from contaminated fruits and vegetables suggest that the pathogens in manure lagoons are either culturable (Ibekwe and Grieve 2003), or in an altered viable but nonculturable state that may or may not be induced by stress (Rigsbee *et al.* 1997; Cuny *et al.* 2005). The pathogens are later resuscitated to a culturable state (Steinert *et al.* 1997) on produce, or become virulent and infectious by *in vivo* resuscitation (Rahman *et al.* 1996) in the host. Hence, it is critical to determine the conditions under which pathogens revert to culturable state. Although, circulating aeration of lagoons did not influence the decline of *E. coli* in this study, it is possible that seasonal, biologi-

cal, and chemical factors might have a greater influence on the fate of pathogens in this environment. Thus, determining the survival factors for pathogens in manure lagoons is crucial in developing strategies to minimize the contamination of food and fodder crops irrigated with wastewater from dairies.

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