

ORIGINAL ARTICLE

Most probable number methodology for quantifying dilute concentrations and fluxes of *Escherichia coli* O157:H7 in surface waters

M.B. Jenkins¹, D.M. Endale¹, D.S. Fisher¹ and P.A. Gay²

¹ USDA-Agriculture Research Service, J. Phil Campbell, Sr., Natural Resource Conservation Center, Watkinsville, GA, USA

² University of Georgia, Tifton, GA, USA

Keywords

baseflow, *Escherichia coli*, faecal enterococci, mass flux, watershed.

Correspondence

Michael B. Jenkins, 1420 Experiment Station Road, Watkinsville, GA 30677, USA.
E-mail: michael.jenkins@ars.usda.gov

2008/0728: received 29 April 2008, revised 9 July 2008 and accepted 28 July 2008

doi:10.1111/j.1365-2672.2008.04028.x

Abstract

Aims: To better understand the transport and enumeration of dilute densities of *Escherichia coli* O157:H7 in agricultural watersheds, we developed a culture-based, five tube-multiple dilution most probable number (MPN) method.

Methods and Results: The MPN method combined a filtration technique for large volumes of surface water with standard selective media, biochemical and immunological tests, and a TaqMan confirmation step. This method determined *E. coli* O157:H7 concentrations as low as 0.1 MPN per litre, with a 95% confidence level of 0.01–0.7 MPN per litre. *Escherichia coli* O157:H7 densities ranged from not detectable to 9 MPN per litre for pond inflow, from not detectable to 0.9 MPN per litre for pond outflow and from not detectable to 8.3 MPN per litre for within pond. The MPN methodology was extended to mass flux determinations. Fluxes of *E. coli* O157:H7 ranged from <27 to >10⁴ MPN per hour.

Conclusion: This culture-based method can detect small numbers of viable/culturable *E. coli* O157:H7 in surface waters of watersheds containing animal agriculture and wildlife.

Significance and Impact of the Study: This MPN method will improve our understanding of the transport and fate of *E. coli* O157:H7 in agricultural watersheds, and can be the basis of collections of environmental *E. coli* O157:H7.

Introduction

Public attention has focussed on animal agriculture as a potential nonpoint source of faecal bacteria and zoonotic pathogens that cause human disease (Ferguson *et al.* 2003; Jamieson *et al.* 2004). By observing elevated concentrations of both total and faecal coliforms, *Escherichia coli*, and faecal enterococci in surface waters, several researchers have implicated animal agriculture as a source of faecal contamination (Walker *et al.* 1990; Edwards *et al.* 1997; Fisher *et al.* 2000). Over the last two decades, several outbreaks of haemorrhagic colitis and haemolytic uremic syndrome have occurred that have been caused by *E. coli* O157:H7 infections from contaminated surface water (Olsen *et al.* 2002) and

groundwater (Hrudey *et al.* 2003), and recently from fresh spinach contaminated with *E. coli* O157:H7 the source of which was attributed to cattle (*Bos taurus*) and feral swine (*Sus scrofa*) (Jay *et al.* 2007). A common reservoir of *E. coli* O157:H7 is cattle, but other agricultural animals (Chapman *et al.* 1997; Kudva *et al.* 1997; Wallace *et al.* 1997), and wildlife such as white-tailed deer (*Odocoileus virginianus*) (Rice *et al.* 2003) and feral swine (Jay *et al.* 2007) are known sources. Faecal shedding of *E. coli* O157:H7 contributes to the pathogen load of watersheds with animal agriculture and wildlife (Ferguson *et al.* 2003; Jamieson *et al.* 2004). Dilute concentrations of *E. coli* O157:H7 in recreational waters may pose a public health risk, given that their infective dose can be as low as 10–100 cells (Willshaw *et al.* 1994;

Jones 1999) and their survival in terrestrial environments can be as long as 80 days (Franz *et al.* 2007).

Attempts to assess the human health risk for surface waters contaminated with microbial pathogens such as *E. coli* O157:H7 have been hampered by a lack of quantitative data such as pathogen concentration (Ferguson *et al.* 2003). We, therefore, developed a culture-based most probable number (MPN) method that would make it possible to determine dilute concentrations of viable/culturable *E. coli* O157:H7 in surface waters of watersheds with animal agriculture and/or wildlife. MPN methods have been developed for quantifying concentrations of *E. coli* O157:H7 in meat products (Chapman *et al.* 2001), and faeces (Fegan *et al.* 2003; Stephens *et al.* 2007), but methods are needed for quantifying dilute concentrations of viable/culturable *E. coli* O157:H7 in environmental surface waters.

As part of a project investigating the effectiveness of impoundments on attenuating baseflow flux of faecal indicator bacteria and pathogens such as *E. coli* O157:H7, we combined a filtration method described by Loge *et al.* (2002), selective media, biochemical and immunological tests for putative identification of *E. coli* O157:H7 described by Rice *et al.* (1996) and recommended in Standard Methods (Clesceri *et al.* 1998), and a TaqMan confirmation step (Sharma 2002).

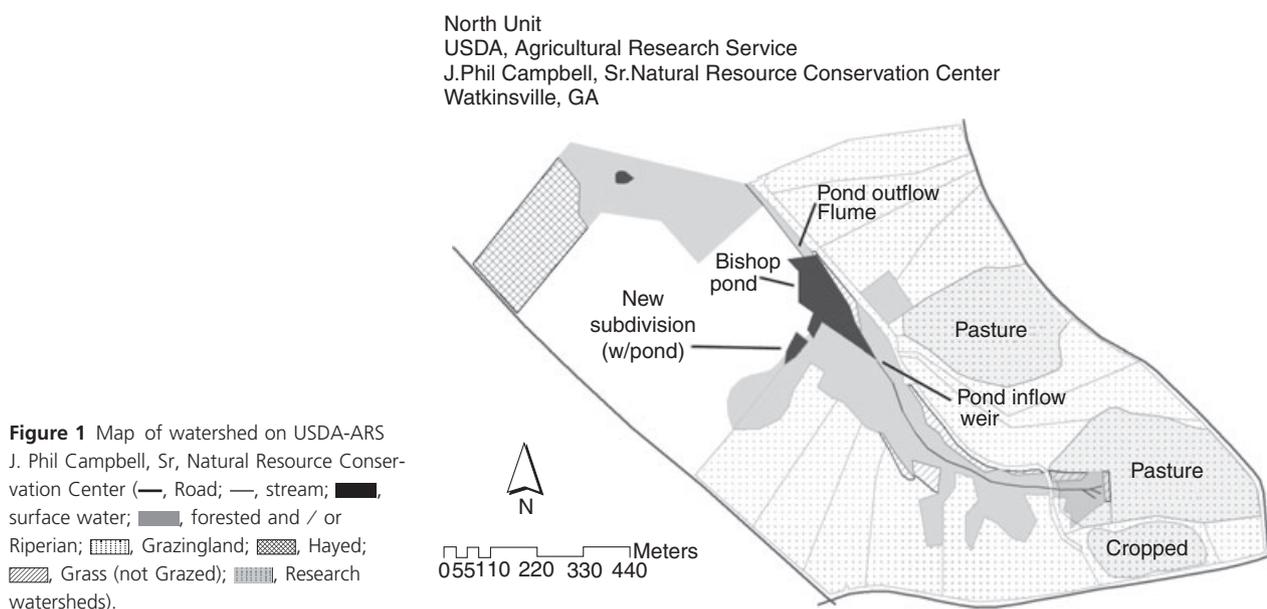
The objective of this research was to develop a culture-based MPN method for determining dilute concentrations of *E. coli* O157:H7 for stream inflow into a pond, the pond's outflow and within the pond. Because regulatory agencies use concentrations of *E. coli* and faecal enterococci to indicate the presence of zoonotic pathogens in

surface waters, we focussed on the relation between baseflow densities and mass fluxes of this zoonotic pathogen, the faecal indicator bacteria and the total aquatic bacterial community. An advantage of this culture-based method in contrast to nonculture-based methods such as PCR is the capacity to develop a culture collection for further study and forensic purposes related to microbial source tracking.

Materials and methods

Study site and sampling scheme

Located at the USDA-ARS J. Phil Campbell, Sr., Natural Resource Conservation Center in the Southern Piedmont of Northeast Georgia, the study site is a small, ~100 ha, watershed that consists of grazed pastures, a cropped (2.7 ha) catchment under conservation tillage that is amended annually with poultry litter, and a wooded riparian zone from which agricultural animals are excluded (Fig. 1). With the exception of the cropped catchment, rotational grazing of a purebred herd of Angus cattle between 500 and 700 head occurred on the paddocks on both sides of the stream flowing into the pond (Fig. 1). These paddocks comprise 56 ha upstream from the pond. No more than 200 head of cattle (cows plus calves) were grazed on a paddock at one time. The average total cattle days (number of cattle \times number of days on pasture) for both years was 2398. Cattle were moved between paddocks every 6 weeks depending on the availability of forage. An unknown number of deer, rodents and other forms of wildlife, a diverse avian



community, and domestic animals from the neighbouring subdivision were potential sources of faecal bacteria. Topographic slopes range from 2% to 10% in the pastures and cropped field. In the riparian zone, stream banks can be as steep as 30%. Bedrock begins 3–30 m below ground surface. Soils belong to the Cecil and Pacolet series (fine, kaolinitic, thermic, Typic Kanhap-ludult). A first-order stream fed by a series of springs flows into and out of a pond that captures base and storm flow from approximately 60% of the 100-ha watershed. The remaining 40% of the watershed that the pond does not serve feeds into the stream below the pond. The bathymetry of the pond was established through GPS/GIS survey, and is approximately 225 m long, and has an average width of 70 m. The deepest part is 4 m from permanent pool level and occupies a 35- by 80-m² area close to the outlet. The bed level gradually rises towards the edges where it is 0.4 m from permanent pool level. The pond holds about 24 000 m³ of water at permanent pool level. Outflow is through a riser and horizontal conduit pipes. A 120° V-notch weir and a 0.46-m H-flume of USDA specification (Brakensiek *et al.* 1979) are used to measure inflow 50 m upstream of the upper end of the pond and at outflow, respectively (Fig. 1). Pond inflow also occurs from a small spring one-third of the way from the upper end of the pond and passes through another small pond before entering the pond, but the small pond is not instrumented for measuring flow (Fig. 1). Differences in measured pond inflow and outflow rates partly indicated the contribution of the small pond. Appropriate calibration curves were used to convert flow head at each measuring device into flow rate. The V-notch weir head was measured with a Campbell Scientific Inc. (Logan, UT, USA) shaft encoder with the float mechanism installed in a stilling well. For the flume, a 17.24-kPa depth sensing transducer (Druck Incorporated, New Fairfield, CT, USA) located in a stilling well of the flume was used. Each sensing device was connected to a Campbell Scientific CR10X data logger programmed to record average flow depth every 5 min. These 5-min flow data were then processed with a computer to produce flows at appropriate time intervals needed for the research. Pond depth was similarly measured with a shaft encoder. Throughout each year, baseflow samples were collected every second Tuesday of each month. Because of logistical considerations, *E. coli* O157:H7 was not enumerated every month. Pond inflow and outflow samples were collected at the lip of the V-notch weir and H-flume, respectively. Pond water samples were taken from the surface at a designated site within the pond. Because of the low concentrations of *E. coli* O157:H7 that Loge *et al.* (2002) reported, 20-l samples

were taken at the inflow and outflow sites and within the pond (Fig. 1). Three pairs of 10-l Nalgene containers, sterilized with 5% commercial hypochlorite and rinsed with ultrapure water, were used for collecting baseflow samples for *E. coli* O157:H7. Sterile 1.5-l Nalgene bottles were used to collect samples for total suspended solids (TSS), faecal indicator bacteria and total direct microbial counts. Subsamples of 100 ml were taken for TSS determination. Because of the previously observed log-normal distribution (Fisher *et al.* 2000), single 0.2- to 0.4-l subsamples were taken for determination of faecal indicator bacteria. Subsamples as 9-ml undiluted aliquots were taken for total direct microbial counts. Flux rates of microbes were determined by the product of microbial concentration (MPN per litre of water) and the rate of the pond inflow and outflow (l h⁻¹) at the time of sampling.

Determination of TSS and microbe concentrations

Total suspended solids

TSS were determined by standard methods (Clesceri *et al.* 1998).

Faecal indicator bacteria

Water samples were assayed for *E. coli* and faecal enterococci with commercial Colilert and Enterolert reagents and Quanti-Tray-2000, respectively (IDEXX Laboratories, Inc., Westbrook, ME, USA). For each pond inflow, outflow and within pond sample, a 10-ml subsample of an appropriate 10-fold dilution was added to 90 ml of Colilert[®] and Enterolert[®] substrate. The inoculated substrate was then poured into a Quanti-Tray[®], sealed, and incubated for 24 h at 35.5°C for Colilert[®] and 24 h at 41°C for Enterolert[®]. With each set of samples, a blank sterile water sample was run. Samples were enumerated by using the methodology described by Budnick *et al.* (1996) and Eckner (1998). Data were expressed as MPN cells 100 ml⁻¹. Samples analysed by Colilert[®] and Enterolert[®] methodology that resulted in no cells detected were considered to have a concentration of at most 0.5 MPN 100 ml⁻¹ of the sample.

Total direct microbial counts

We combined methods described by Kepner and Pratt (1994) and Clesceri *et al.* (1998) for determining total direct microbial counts. To 9 ml of each environmental water sample, 1 ml of 10% (w/v) phosphate-buffered glutaraldehyde was added as fixative. To disperse the microbial suspension, 1.1 ml of dispersant [0.1 mol l⁻¹ tetrasodium pyrophosphate (Sigma Chemical Co., St Louis, MO, USA)] was added to the 10 ml of fixed sample and sonicated for 30 s with a 1-mm sonication

probe (Cole Parmer, Vernon Hills, IL, USA). Aliquots of fixed and dispersed samples were stained with 5 μl of a stock solution (2.0 $\mu\text{g ml}^{-1}$) of 4',6-diamidino-2-phenylindole [DAPI (Sigma Chemical Co., St Louis, MO, USA)] ml^{-1} of sample and incubated at 4°C in the dark for 60 min. Depending on the assumed concentration of total micro-organisms in the samples, between 1 and 4 ml of sample were filtered under <4.0 kPa vacuum with a blackened 25-mm diameter, 0.2- μm pore size polycarbonate membrane filter (Millipore, Bedford, MA, USA); this was followed by 1–4 ml of water to remove excess stain. The filter was then mounted on a drop of low-fluorescence immersion oil on an acetone-cleaned microscope slide and 10 μl of Citifluor Antifadent Mounting Medium AF3 (Electron Microscopy Sciences, Hatfield, PA, USA) was placed on the filter, and covered with a 22-mm cover slip. The slide was examined with a Leica DMR fluorescent microscope equipped with a filter to observe DAPI-stained cells and a 100 \times /1.40-0.7 oil PL APO DIC (differential interference contrast) objective with 10 \times eye pieces. Twenty fields (out of >10⁵ possible fields) were brought into view and counted. Total direct counts were determined by the equation:

$$\text{Cells ml}^{-1} = (N \times A_t) / (V_f \times A_g)$$

where N is the mean cell count, A_t is the area of the filter, V_f is the volume of sample filtered and A_g is area of the field.

MPN method for *Escherichia coli* O157:H7 determination

Escherichia coli O157:H7 was quantified by combining a modified concentration method described by Loge *et al.* (2002) with standard cultural methods, and a TaqMan confirmation step. A 293-mm, 1- μm pore size FALP filter (Millipore, Bedford, MA, USA) was prewetted in methanol to make it hydrophilic, and sealed in a custom-made filter holder that was attached to a vacuum pump. Sterile water was pumped through it to remove methanol before the 20 l of sample was pulled through it. The filter was eluted by scrubbing with a sterile stiff brush for 10 min as Loge *et al.* (2002) described. Eluted extract was collected in 50-ml centrifuge tubes, centrifuged at 10 000 g for 25 min, resuspended in PBS to consolidate the extracted material and centrifuged again. The pellet was resuspended in 5 ml of PBS, and 1-ml aliquots were used to inoculate the first five tubes containing 9 ml of Laural Tryptose broth [LTB (Becton, Dickinson and Company, Franklin Lakes, NJ, USA)] followed by three 10-fold dilutions to complete a five-tube,

four by 10-fold dilution scheme. The inoculated LTB was incubated at 35°C for 24 h. Sorbitol MacConkey [SMAC (Becton, Dickinson and Company, Franklin Lakes, NJ, USA)] agar plates were inoculated with 10–100 μl of LTB showing growth and streaked for colony isolation and purity and incubated at 35°C for 24 h. Three to six colourless colonies were picked and streaked for isolation and purity on Beef Heart Infusion [BHI (Becton, Dickinson and Co., Franklin Lakes, NJ, USA)] agar plates which were incubated at 35°C for 24 h. A colony from each BHI plate was then inoculated into LTB-4-methylumbelliferyl- β -D-glucuronide (LTB-MUG) broth (Becton, Dickinson and Co.) in tubes with Durham tubes for gas collection. LTB-MUG tubes positive for *E. coli* O157:H7 did not fluoresce and showed gas production. LTB-MUG positive isolates were then tested for glutamate decarboxylase (GAD) activity as described by Rice *et al.* (1993), and latex agglutination with the Oxoid Dryspot *E. coli* O157 Test Kit (Oxoid Ltd, Cambridge, UK). If both GAD and latex agglutinations appeared positive, then confirmation was made with a TaqMan assay on a colony from a respective BHI plate. Primers and Probe were specific for *eaeA* gene (Sharma 2002): *eaeA* forward 5'-GTAAGTTACTACTATAAAAAGCACCGTC-3', *eaeA* reverse 5'-TCTGTGTGGATGGTAATAAATTTTTG-3', and the TaqMan *eaeA* probe 5'-AAATGGACATAGCATCAGCATAATAGGCTTGCT-3' with reporter dye FAM conjugated at the 5' end and quencher dye TAMRA conjugated at the 3' end. Amplification was undertaken with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and under conditions set by Sharma (2002). A replicate LTB tube of a particular dilution was considered positive for *E. coli* O157:H7 if at least one isolate from that tube was positive for GAD, latex agglutination, and had a positive fluorescent threshold cycle in the TaqMan assay (Sharma 2002). MPN and 95% confidence limits (95% CL) of *Escherichia coli* O157:H7 cells were calculated by the number of positive tubes with all determinations having improbability ratios greater than 0.0001 [Briones and Reichardt 1999; Garthright and Blodgett 2003; Blodgett 2005; <http://www.cfsan.fda.gov/~ebam/bam-a2.html> (accessed 25 April 2008)].

To determine the extraction efficiency of the filtration method previously described by Loge *et al.* (2002), 10–20 l grab samples of stream water (taken from the pond inflow site) were spiked with 300–900 cells of *E. coli* O157:H7 #G5244 (Obtained from USDA-FSIS, Food Emergency Response Network Division, Athens, GA, USA) as determined by serial dilution plate counts on BHI agar. These spiked samples were filtered through the 293-mm FALP filter, eluted, and processed as described above.

Data analysis

Significant differences ($P \leq 0.05$) between pond inflow and outflow natural log-transformed concentrations of *E. coli*, and faecal enterococci were analysed with Proc Mixed of SAS (ver. 9.1) (SAS Institute, Inc., Cary, NC, USA) and the program's repeated measures option and treating sampling time as the repeated measure (Littell *et al.* 1996).

Results

Results from filtering pond water spiked with known numbers of *E. coli* O157:H7 through the FALP filter indicated a range of recovery between 50% and 61%. The baseflow TSS data indicated a significant difference between pond inflow and within pond and pond outflow (Table 1). Although samples for TSS determinations were not collected and performed for the spiked filtration efficiency tests, they were expected to fall within the range of TSS concentrations for the pond inflow samples. All isolates obtained from positive LTB tubes were positive for GAD, latex agglutination and TaqMan assay. The MPN method for *E. coli* O157:H7 confirmed the presence of small numbers of this zoonotic pathogen (Table 2) in the inflow and outflow of a pond, and within the pond situated near the head of a watershed containing animal agriculture and wildlife (Fig. 1). Not all of the isolates from an LTB tube that was positive for both GAD and latex agglutination were confirmed by the TaqMan assay to be *E. coli* O157:H7. *Escherichia coli* O157:H7 was enumerated 6 out of the 10 sampling times, and specifically on four, one and four of the sampling times for pond inflow, outflow and within pond, respectively (Table 2). No pond inflow and outflow concentrations were measured at the same sampling time. On two sampling dates (May 2006 and February 2007) *E. coli* O157:H7 was enumerated in both pond inflow and within pond samples. On one sampling date (July 2006) concentrations of *E. coli* O157:H7 were determined for within pond and pond outflow samples. The MPN determinations ranged from below detection limit (<0.01 MPN per litre) to 9.0 MPN per litre for

pond inflow samples, from below detection limit to 0.9 MPN per litre for pond outflow samples and from below detection limit to 8.3 MPN per litre for within pond samples. Concentrations of both faecal indicator bacteria were greater ($P < 0.01$) in the inflow than in the pond outflow or within pond samples, and total counts (TC) of pond inflow were less than TC for within pond and pond outflow ($P < 0.01$) (data not shown).

At sampling times, volumetric flow rates at pond inflow were generally less than those at the pond outflow (Table 3). The combination of pond volumetric inflow and outflow rates at times of sampling with their respective concentrations of *E. coli* O157:H7 gave estimates of mass flux rates. The pond inflow flux rate of *E. coli* O157:H7 (Table 3) for November 2006, and February and May 2007 were estimated to be $>10^3$ to 10^4 cells per hour. The outflow flux of *E. coli* O157:H7 for July, 2006 was estimated to be greater than 10^3 cells per hour. If concentrations of *E. coli* O157:H7 were just below the limit of detection, then the potential flux of *E. coli* O157:H7 could range between <10 and 100 cells per hour.

Discussion

The difference between volumetric flow rates at pond inflow and outflow were partly attributable to subsurface contributions around the pond and from the small spring-pond combination entering the pond from the west originating in a housing development (Fig. 1).

The filtration recovery efficiencies were within the range that Loge *et al.* (2002) reported for this method. Although both TSS and TC concentrations for within pond and pond outflow samples were greater than TSS and TC concentrations of pond inflow samples, these environmental factors did not appear to affect the filter's capacity for 20 l of sample, and appeared not to affect recovery of *E. coli* O157:H7. Baseline sensitivity for this MPN method was based on the MPN for one positive tube at the lowest dilution of a five-tube dilution series, and was considered to be the most likely low value of the 95% CL (Garthright and Blodgett 2003): 0.01 MPN per litre. Variables controlling the method's detection limits included volume of the sample passed through the FALP filter (V_f), recovery of organisms (R), baseline sensitivity (S), fraction of volume of filter concentrate used to inoculate the first replicate tubes of LTB (which was always 1.0 ml) and an inhibition factor (I) that could prevent the selection and confirmation of *E. coli* O157:H7 by the TaqMan assay, and is expressed as the inverse of the highest dilution with a positive tube (Loge *et al.* 2002). Analogous to the equation for determining the detection limit of the PCR-based method that Loge *et al.* (2002)

Table 1 Means (\pm SD) of total suspended solids determined for baseflow water samples of pond inflow, outflow and within pond for the sampling times between September 2006 and December 2007

Water sample	mg l ⁻¹
Inflow	1.53 (\pm 1.19)*
Pond	7.86 (\pm 3.55)**
Outflow	9.61 (\pm 5.57)**

*, ** Indicate a significant difference at $P < 0.01$.

Table 2 MPN determinations for *E. coli* O157:H7 compared with the faecal indicator bacterial *E. coli* (EC) and faecal enterococci [FE] and total direct microbial counts {TC} baseflow concentrations in Bishop Pond inflow, within pond and outflow

Sampling time	MPN l ⁻¹ (95% CL MPN 100 ml ⁻¹) [MPN 100 ml ⁻¹]* {cells ml ⁻¹ }†		
	Inflow	Pond	Outflow
May 2006	0.4 (0.001–0.070) [132, 72] {1.75E6}	0.9 (0.013–0.64) [10, 4] {2.43E6}	ND‡ [10, ND] {1.18E6}
July 2006	ND [345, 301] {1.36E6}	0.1 (0.001–0.07) [6, 2] {2.60E6}	0.9 (0.013–0.64) [ND, ND] {2.90E6}
September 2006	ND [148, 341] {4.04E5}	ND [15, 10] {2.06E6}	ND [ND, 20] {1.88E6}
November 2006	9.0 (0.13–6.4) [74, 328] {4.50E5}	ND [7, 1] {6.60E5}	ND [10, ND] {8.20E5}
February 2007	0.35 (0.02–0.06) [121, ND] {1.20E6}	8.3 (0.30–2.20) [36, 2] {4.20E6}	ND [20, ND] {4.00E6}
May 2007	2.0 (0.03–1.40) [122, 181] {4.20E5}	ND [5, 2] {7.41E6}	ND [10, ND] {6.33E6}
June 2007	ND [211, 253] {7.78E6}	ND [2, 6] {1.06E7}	ND [ND, 181] {1.06E7}
August 2007	ND [794, 957] {1.97E6}	ND [5, 4] {1.51E7}	ND [ND, ND] {6.47E6}
September 2007	ND [776, 1005] {7.44E7}	ND [4, 4] {9.60E7}	ND [10, 10] {5.53E7}
November 2007	ND [613, 708] {3.90E6}	0.9 (0.013–0.64) [27, ND] {1.86E7}	ND [10, ND] {1.18E7}

*MPN determinations of EC and FE 100 ml⁻¹, respectively.

†TC.

‡ND means not detected or <0.001 100 ml⁻¹ for *E. coli* O157:H7 and <0.5 100 ml⁻¹ for faecal indicator bacteria.

Table 3 Flux of *Escherichia coli* O157:H7 in pond inflow and outflow and corresponding hydrologic flux of pond inflow and outflow

Sampling date	Flux of <i>E. coli</i> O157:H7 (log ₁₀ MPN per hour)		Hydrologic flux (l s ⁻¹)	
	Inflow	Outflow	Inflow	Outflow
May 2006	3.72	<2.34*	3.68	6.12
July 2006	<1.77	3.74	1.70	1.70
September 2006	<1.88	<0.67	2.11	0.13
November 2006	4.83	<1.80	2.11	1.75
February 2007	3.69	<2.40	3.93	7.04
May 2007	4.09	<2.20	1.70	4.44
June 2007	<1.77	<1.89	1.70	2.14
August 2007	<1.44	<1.52	0.76	0.91
September 2007	<1.44	<1.59	1.76	1.09
November 2007	<1.66	<1.94	1.27	2.42

*Flux calculations with 'less than' symbol are based on detection limit of <0.001 MPN 100 ml⁻¹.

presented, the overall detection limit of the MPN method can be expressed as

$$\text{Detection limit} = (IS)/(RV_f)$$

Thus, the overall detection limit was highly dependent on the potential inhibition factor (*I*) because it ranged from 10 to 1000; whereas the recovery factor (*R*) and volume of sample (*V_f*) had smaller ranges and had relatively little effect on the range of overall detection limit (i.e., 0.01–1 MPN per litre). Given the range of extraction efficiency, variables affecting detection limits, and the probability of *E. coli* O157:H7 cells being components of biofilms or particulate aggregates, our MPN determinations were likely underestimations.

Of the detectable concentrations of *E. coli* O157:H7 in the pond inflow, only the May 2006 density occurred with an *E. coli* concentration greater than the criteria USEPA established for a single allowable estimate of microbial density; whereas three of the detectable concentrations of *E. coli* O157:H7 (May 2006, November 2006 and May 2007) occurred with faecal enterococci concentrations greater than USEPA's allowable estimate of microbial density. The criteria for *E. coli* and faecal enterococci ranged from 126 to 575 100 ml⁻¹, and 35 to 501 100 ml⁻¹, respectively, depending on the specific criterion (USEPA 1986). On the other hand, concentrations of *E. coli* O157:H7 within the pond and in pond outflow occurred with faecal indicator bacteria that were either not detectable, or below USEPA's allowable estimate of microbial density, and was probably the result of a direct contribution from *E. coli* O157:H7 shedding wildlife to the pond, or an indication of a negative binomial distribution of *E. coli* O157:H7. Although the total direct microbial counts for within pond and pond outflow were greater than the inflow counts, and pond outflow concentrations of the faecal indicator bacteria were either not detectable, or less than USEPA's allowable microbial density, and less than pond inflow concentrations, no such consistent short-term pattern was associated with the dilute concentrations observed for *E. coli* O157:H7. The apparent lack of connection between pathogenic *E. coli* O157:H7 and the two standard faecal indicator bacteria underscore the necessity for methods capable of enumerating pathogens themselves.

We adapted the method for detecting *E. coli* O157:H7 in environmental waters that Rice *et al.* (1996) developed and made it quantitative by putting it into an MPN format. Sensitivity of the method was increased by adapting

the filtration method of Loge *et al.* (2002); and biochemical and immunological assays for confirming the identity of *E. coli* O157:H7 were replaced with the TaqMan assay of Sharma (2002). Other methods have been developed to enumerate *E. coli* O157:H7 in surface waters. Combining a 100-fold concentration of raw surface water with an immunomagnetic bead-electrochemiluminescence methodology of enumeration, Shelton and Karns (2001) reported a detection limit for *E. coli* O157:H7 of 25 cells 100 ml⁻¹. Ibekwe *et al.* (2002) reported a detection limit of 6.4×10^3 *E. coli* O157:H7 cells per millilitre of environmental surface water with a real-time, quantitative PCR method.

Baseflow fluxes of *E. coli* O157:H7 have not been investigated systematically partly because of the lack of methods for enumerating dilute concentrations of *E. coli* O157:H7. In addition to determining concentrations of *E. coli* O157:H7 in surface waters of watersheds impacted by animal agriculture and wildlife, the fact that our baseflow fluxes of *E. coli* O157:H7 ranged between 10^3 and 10^4 viable cells per hour may further help to establish the level of risk this pathogen could have on public health.

The culture-based MPN method described and tested in this study has quantified dilute densities of *E. coli* O157:H7 in environmental surface water of a watershed containing animal agriculture and wildlife. It provides a quantitative tool for improving our understanding of the fluxes of *E. coli* O157:H7 in association with the faecal indicator bacteria *E. coli* and faecal enterococci, and total aquatic microbial community. In systems such as those described here, where flow rate is measured continuously, more frequent flux data can be developed and correlated with the management on watersheds upstream with respect to cattle density and movement and/or cropping schedule. From these data, one might develop best management practices that would lower incidences of high fluxes of these contaminants into surface waters. This method of quantifying densities of *E. coli* O157:H7 in environmental surface waters appears to be more sensitive than the method Shelton and Karns (2001) reported, and nonculture-based PCR methods (Ibekwe *et al.* 2002; Loge *et al.* 2002), and can provide the added advantage of having the capacity to develop collections of environmental *E. coli* O157:H7.

Acknowledgements

The authors wish to express their appreciation to S. Humayoun, S. Norris, J. Sterling and S. Steed for their expert technical assistance. This study was supported in part by a grant from the USDA-CSREES National Research Initiative Competitive Grants Program.

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