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ORIGINAL ARTICLE

Most probable number methodology for quantifying dilute concentrations and fluxes of *Salmonella* in surface waters

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Abstract

Aims: To better understand and manage the fate and transport of *Salmonella* in agricultural watersheds, we developed a culture-based, five tube–four dilution most probable number (MPN) method for enumerating dilute densities of *Salmonella* in environmental waters.

Methods and Results: The MPN method was a combination of a filtration technique for large sample volumes of environmental water, standard selective media for *Salmonella* and a TaqMan confirmation step. This method has determined the density of *Salmonella* in 20-l samples of pond inflow and outflow streams as low as 0·1 MPN l^{-1} and a low 95% confidence level 0·015 MPN l^{-1}. *Salmonella* densities ranged from not detectable to 0·55 MPN l^{-1} for pond inflow samples and from not detectable to 3·4 MPN l^{-1} for pond outflow samples. *Salmonella* densities of pond inflow samples were associated with densities of *Escherichia coli* and faecal enterococci that indicated stream contamination with faeces and with nondetectable pond outflow densities of the faecal indicator bacteria. The MPN methodology was extended to flux determinations by integrating with volumetric measurements of pond inflow (mean flux of 2·5 l s^{-1}) and outflow (mean flux of 5·6 l s^{-1}). Fluxes of *Salmonella* ranged from 100 to greater than 10^4 MPN h^{-1}.

Conclusions: This is a culture-based method that can detect small numbers of *Salmonella* in environmental waters of watersheds containing animal husbandry and wildlife.

Significance and Impact of the Study: Applying this method to environmental waters will improve our understanding of the transport and fate of *Salmonella* in agricultural watersheds, and can be the basis of valuable collections of environmental *Salmonella*.

Introduction

Public attention has focused on animal husbandry as a potential nonpoint source of faecal bacteria and zoonotic pathogens that cause human disease (Coyne and Blevins 1995; Edwards *et al.* 1997; Ferguson *et al.* 2003; Jamieson *et al.* 2004). By observing elevated concentrations of both total and faecal coliforms, *Eschericia 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). *Salmonella* is a cause of gastroenteritis and a leading cause of food-related deaths (Mead *et al.* 1999). Prevalence for shedding *Salmonella* in faeces is associated with beef and dairy cattle, poultry and swine (Pell 1997; Davies *et al.* 1999). Shedding of *Salmonella* in faeces can contribute to the pathogen load of watersheds by runoff from fields with animal husbandry (Ferguson *et al.* 2003; Jamieson *et al.* 2004). Dilute concentrations of *Salmonella* in recreational waters may pose a public health risk given that their infective dose can be as low as 100

cells (Bitten 1994) and that the frequency of multi-drug-resistant strains of *Salmonella* has increased (Swartz 2002; Cobbold *et al.* 2006).

Attempts to assess the human health risk for surface waters contaminated with microbial pathogens such as *Salmonella* have been hampered by a lack of quantitative data such as pathogen concentration (Ferguson *et al.* 2003). The objective of this study was to develop a culture-based most probable number (MPN) method that would make it possible to determine dilute concentrations of *Salmonella* in surface waters of watersheds with animal husbandry and wildlife. Although MPN methods for quantifying concentrations of *Salmonella* in surface waters of watersheds impacted by animal husbandry have been developed (Baudart *et al.* 2000; Ho and Tam 2000; Lemarchant and Lebaron 2003), the apparent limit of detection of these methods ranged between 0·6 and 1 cell l^{-1}. As part of a project investigating the effectiveness of impoundments on attenuating baseflow flux of faecal indicator bacteria and *Salmonella*, we combined a filtration method described by Loge *et al.* (2002), selective media for isolating *Salmonella* recommended by Standard Methods (Clesceri *et al.* 1998) and a TaqMan confirmation step (Carlson *et al.* 1999).

The aim of this study was to develop a culture-based MPN method for determining dilute concentrations of *Salmonella* for stream inflow into a pond and the pond's outflow, and observe the relationship between baseflow densities of this zoonotic pathogen, the faecal indicator bacteria and the total aquatic bacterial community. An advantage to this culture-based method in contrast to noncultural 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 animals are excluded (Fig. 1). 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 are Cecil and Pacolet (fine, kaolinitic, thermic and Typic Kanhapludult). A first-order stream fed by a series of springs flows into and out of a pond (Bishop Pond) that serves approx. 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 approx. 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 m^2 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 m^3 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 spring one-third of the way from the upper end of the pond and passes through another small pond, but because this small pond is on private property

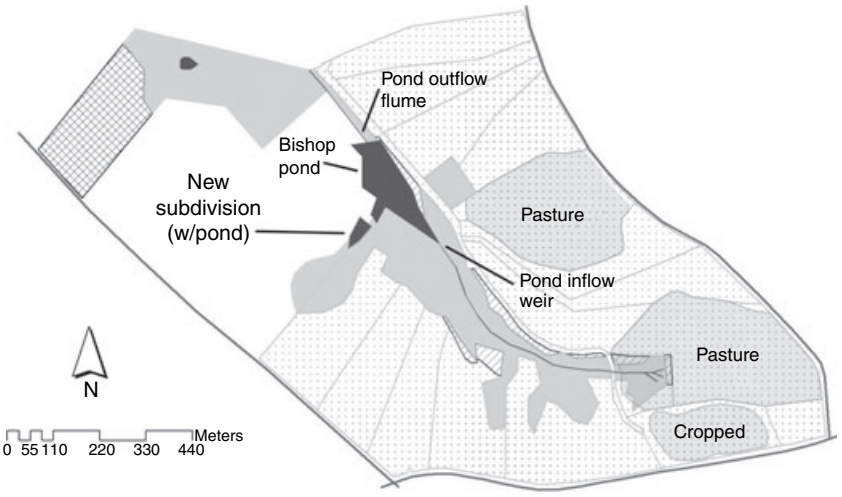

Figure 1 Map of watershed on USDA-ARS J. Phil Campbell, Sr., Natural Resource Conservation Center. ▬, road; —, stream; ■, surface water; ▩, forested and/or riparian; ▨, grazingland; ▩, hayed; ▨, grass (not grazed); ▨, research watersheds.

we have not been able to instrument it for measuring flow (Fig. 1). 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 flow depth sensing Druck Incorporated (New Fairfield, CT, USA) transducer located in a stilling well of the flume was used. Each flow sensing device is 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 the computer to produce flows at appropriate time intervals needed for the research. Pond depth was similarly measured with a shaft encoder. Based on the assumed low *Salmonella* concentration and results reported by Loge *et al.* (2002), 20-l samples were taken at the inflow and outflow sites (Fig. 1). Because of the previously observed log-normal distribution, single 0·2- to 0·4-l samples were taken for faecal indicator bacteria (Fisher *et al.* 2000). Subsamples as 9-ml undiluted aliquots were taken for total direct microbial counts.

Determination of microbe concentrations

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). Samples were enumerated using the methodology described by Budnick *et al.* (1996) and Eckner (1998). Data were expressed as MPN cells 100 ml^{-1}.

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 sample were stained with 5 μl of a stock solution (2·0 μg 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 microorganisms 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×/1·40–0·7 oil PL APO differential interference contrast (DIC) objective with 10× eye pieces. Twenty fields (out of $>10^5$ possible fields) were brought into view and counted. Total direct counts were determined by the equation:

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

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 the area of the field.

MPN method for *Salmonella* determination

Salmonella were 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) 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 tetrathionate broth (TB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) followed by three 10-fold dilutions to complete a five-tube, and four by 10-fold dilution scheme. The inoculated TB was incubated at 35°C for 24 h. Brilliant Green (BG; Becton, Dickinson and Company) agar plates were inoculated with 10–100 μl of TB showing growth and streaked for colony isolation and purity and incubated at 35°C for 24 h. Three to six pink to pinkish white colonies on a red background were picked and streaked for isolation and purity on Beef Heart Infusion (BHI; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) agar plates which were incubated at 35°C for 24 h. A colony from each BHI plate was then stabbed into slants of Lysine Iron agar (LIA;

Becton, Dickinson and Company) and Triple Sugar Iron (TSI; Becton, Dickinson and Company) agar which were incubated at 35°C for 24 h. If both LIA and TSI slants appeared positive for *Salmonella*, then confirmation was made with a TaqMan assay on a colony from a respective BHI plate. Primers and Probe were specific for a 250-bp fragment of the junction between virulence genes *Sip*B and *Sip*C (Carlson *et al.* 1999): SipB/C forward 5′-ACAG-CAAAATGCGGATGCTT-3′ and SipB/C reverse 5′-GCG-CGCTCAGTGT-AGGACTC-3′. The TaqMan probe for *Salmonella*, SipB/C probe was 5′-CCAGGTCACTGACTT-TACTGCTGCTAATACCAA-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) and under conditions set by Sharma and Carlson (2000). MPN and 95% confidence limits (CL) of *Salmonella* cells were calculated by the number of positive tubes with all determinations having improbability ratios >0·0001 (Briones and Reichardt 1999; Garthright and Blodgett 2003; Blodgett 2005; http://www.cfsan.fda.gov/~ebam/bam-a2.html, accessed 1 October 2007).

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 250–700 cells of *Salmonella enterica* serovar Newport ATCC No. 13076 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 ($P \leq 0{\cdot}05$) between pond inflow and outflow natural log-transformed concentrations and fluxes of *E. coli* and faecal enterococci were analysed with Proc Mixed of SAS (Version 9.1; SAS Institute, Cary, NC) and the program's repeated-measures option and treating sampling time as the repeated measure (Littell *et al.* 1996). Regression analyses that were undertaken on natural log-transformed values of pond inflow and outflow concentrations and fluxes of *Salmonella* and faecal indicator bacteria were tested for linearity and slope with a one-way analysis of variance as described by Zar (1999).

Results

Results from filtering pond water spiked with known numbers of *Salmonella* through the FALP filter indicated a range of recovery between 50% and 70%. The MPN method for *Salmonella* confirmed the presence of small numbers of this zoonotic pathogen (Table 1) in the inflow and outflow of a pond at the head of a watershed containing animal husbandry and wildlife. The MPN determinations ranged from below detection limits (<0·015 MPN l^{-1}) to 0·55 MPN l^{-1} for inflow samples and from below detection limits to 3·4 MPN l^{-1} for outflow samples.

The hydrologic flux rates of pond inflow and outflow (Table 2) indicated a greater outflow than inflow. Combining the pond inflow and outflow rates at times of sampling with the concentrations of *Salmonella*, faecal indicator bacteria and total direct microbial counts gave estimates of the flux rates for these microbial populations and communities. The pond inflow flux rate of *Salmonella* (Table 3) for October and December 2006 was estimated to be $>10^3$ cells h^{-1}. The outflow flux of *Salmonella* for August 2006 was estimated to be $>10^4$ cells h^{-1}. If concentrations of *Salmonella* were just below the limit of detection, then the potential flux of *Salmonella* could be >100 cells h^{-1}. Nevertheless, one-way analyses of variance of regressions indicated no significant ($P \leq 0{\cdot}05$) correlations between concentrations and fluxes of *Salmonella*, faecal indicator bacteria and total microbial concentrations. Flux ratios between *Salmonella*, *E. coli*, faecal enterococci and total direct microbial counts for pond inflow and outflow varied between sampling dates (Table 4).

Table 1 Most probable number (MPN) determinations for *Salmonella* compared with *Escherichia coli* (EC), faecal enterococci (FE) and total direct microbial counts (TC) baseflow concentrations in Bishop Pond inflow and outflow

	MPN l^{-1} (95% CL MPN 100 ml^{-1}) [MPN 100 ml^{-1}]* {cells ml^{-1}}†	
Sampling time	Inflow	Outflow
August 2006	0·10 (0·002–0·07) [782; 1182] {2·42E5}	3·4 (0·11–1·10) [ND‡; ND] {1·43E6}
October 2006	0·20 (0·01–0·03) [119; 83] {4·52E5}	ND [ND; ND] {9·10E5}
December 2006	0·55 (0·02–0·15) [246; 63] {5·57E5}	ND [10; ND] {4·57E6}
March 2007	ND [85; 51] {5·44E5}	ND [30; ND] {3·66E6}
May 2007	0·10 (0·002–0·07) [122; 181] {4·23E5}	ND [10; ND] {6·33E6}

*MPN determinations of EC and FE 100 ml^{-1}, respectively.
†TC.
‡ND means not detected or <0·002 100 ml^{-1} for *Salmonella* and <0·5 100 ml^{-1} for faecal indicator bacteria.

Table 2 Hydrologic characteristics of pond inflow and outflow at time of sampling

	Flux rate (l s^{-1})	
Sampling time	Inflow	Outflow
August 2006	1·94	4·18
October 2006	1·47	3·83
December 2006	3·57	7·30
March 2007	3·81	8·10
May 2007	1·70	4·44

Table 3 Flux of *Salmonella* in pond inflow and outflow

	Log_{10} MPN h^{-1}	
Sampling time	Inflow	Outflow
August 2006	2·84	4·71
October 2006	3·03	<2·44*
December 2006	3·85	<2·72
March 2007	<2·43	<2·77
May 2007	2·79	<2·50

*Flux calculations with 'less than' symbol is based on detection limit of <0·002 MPN 100 ml^{-1}.

Discussion

The difference between pond inflow and outflow fluxes was 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. 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): 0·015 MPN l^{-1}. Variables controlling the method's detection limits include volume of 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 tetrathionate (which was always 1·0) and an inhibition factor (I) that could prevent the selection and confirmation of *Salmonella* 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) 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 l^{-1}). Considering the range of extraction efficiency, baseline sensitivity, variables that affect detection limits and the probability that *Salmonella* can be aggregated by adhering to detritus or being components of biofilms, our MPN determinations are likely to be underestimations.

In relation to the faecal indicator bacteria, pond inflow concentrations of *Salmonella* were associated with *E. coli* and faecal enterococci concentrations that were greater than the criteria EPA established for a single allowable estimate of microbial density (Anon 1986). 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 (Anon 1986). On the other hand, the largest concentration of *Salmonella* was associated with the August 2006 outflow sample for which the faecal indicator bacteria were not detectable and was probably the result of a direct contribution from

Table 4 Flux ratios between *Salmonella* (SA), *Escherichia coli* (EC), faecal enterococci (FE) and total direct microbial counts (TC) for pond inflow and outflow at time of sampling

	SA : EC		SA : FE		SA : TC	
Sampling time	Inflow	Outflow	Inflow	Outflow	Inflow	Outflow
August 2006	1 : 7·9E4	1 : 1·5†	1 : 1·2E5	1 : 1·5†	1 : 2·4E9	1 : 4·2E8
October 2006	1 : 5·9E5	1 : 2·5E2‡	1 : 4·1E3	1 : 2·5E2‡	1 : 2·2E9	1 : 4·6E10*
December 2006	1 : 4·5E3	1 : 5·0E3*	1 : 1·1E3	1 : 2·5E2‡	1 : 1·0E9	1 : 2·3E11*
March 2007	1 : 1·9E4*	1 : 1·5E4*	1 : 1·2E4*	1 : 2·5E2‡	1 : 1·2E10*	1 : 1·8E11*
May 2007	1 : 1·2E4	1 : 5·1E3*	1 : 1·8E4	1 : 2·5E2‡	1 : 4·2E9	1 : 3·2E11*

*Ratio based on detection limit values for *Salmonella* at <0·002 MPN 100 ml^{-1}.
†Ratio based on detection limit values for EC and faecal enterococci at <0·5 MPN 100 ml^{-1}.
‡Ratio based on detection limit values for both *Salmonella* and faecal indicator bacteria.

Salmonella shedding wildlife to the pond. Although the total direct microbial counts for pond inflow were nearly an order of magnitude less than the outflow counts (Table 1) and outflow concentrations of the faecal indicator bacteria were either not detectable or significantly less than inflow concentrations (at $P < 0{\cdot}0001$; data not shown), no such consistent short-term pattern was associated with the dilute concentrations observed for *Salmonella*.

Baudart *et al.* (2000) reported on the fluxes of faecal coliforms and *Salmonella* in a watershed with both point and nonpoint sources of these faecal bacteria and focused on sewage discharges and storm events. Lemarchant and Lebaron (2003) reported on fluxes of *Salmonella* from wastewater treatment plants. Both of these studies considered not only concentrations of *Salmonella* but also their daily fluxes because the fluxes over time impact natural ecosystems. Baseflow fluxes of *Salmonella* in contrast to faecal indicator bacteria and total microbial counts have not been investigated systematically partly because of the lack of methods for enumerating dilute concentrations of *Salmonella*. Because some baseflow flux ratios between *Salmonella*, *E. coli* and faecal enterococci are based on their respective detection limits (Table 4), they may not be accurate. The ratio of baseflow *Salmonella* to concomitant fluxes of *E. coli*, faecal enterococci and the total aquatic bacterial community (Table 4) may, however, indicate a connection between *Salmonella* and these bacterial communities that is more indicative of their impact on the ecology of the watershed and its risk to public health.

In order to estimate microbial numbers with maximum caution and approach the system with 'a worst-case scenario', some of the data as noted in Table 4 were adjusted upward based on the limit of detection for *Salmonella*, *E. coli* and faecal enterococci. The ratio of *Salmonella* to *E. coli* ranged between 1 : 1·5, when a measurable flux of *Salmonella* was determined and its corresponding flux of *E. coli* was based on its detection limit, and $1 : 10^5$ when fluxes of *Salmonella* were low or below detection and fluxes of *E. coli* were greater than the USEPA (Anon 1986) criteria of water impairment. A similar pattern was observed between fluxes of *Salmonella* and fluxes of faecal enterococci. Further study is needed to understand the ecological significance of the baseflow flux ratios between *Salmonella*, faecal indicator bacteria and total microbial counts which ranged from $1 : 10^8$ to $1 : 10^{11}$ for inflow and outflow fluxes, respectively.

The culture-based MPN method described and tested in this study has quantified dilute densities of *Salmonella* in environmental surface water of a watershed containing animal husbandry and wildlife. It provides a quantitative tool for improving our understanding of the fluxes of *Salmonella* 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 *Salmonella* in environmental surface waters appears to be more sensitive than non-culture-based PCR methods (Loge *et al.* 2002) and can provide the added advantage of having the capacity to develop collections of environmental *Salmonella* isolates.

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