

## Effect of alternative on-site wastewater treatment on the viability and culturability of *Salmonella choleraesuis*

J. W. Pundsack, R. E. Hicks and R. P. Axler

### ABSTRACT

The objective of this study was to determine how alternative on-site wastewater treatment systems (i.e. subsurface flow constructed wetlands, intermittent sand filters and intermittent peat filters) affect the viability and culturability of *Salmonella choleraesuis* (serotype typhimurium, ATCC 23567). Influent was a high strength septic tank effluent ( $BOD_5$  240–344 mg L<sup>-1</sup>, TN ~ 100 mg L<sup>-1</sup>, TP ~ 15 mg L<sup>-1</sup>) at the Natural Resources Research Institute's (NRR) alternative treatment system test facility in northern Minnesota. Treatment systems were inoculated with cultures of *S. choleraesuis* for 5–7 consecutive days in summer and winter during 1998–99. After the seeding, outflow samples were taken until *Salmonella* counts were sustained at background levels. In addition to culture-based enumeration, *S. choleraesuis* abundances were also measured using fluorescent *in situ* hybridization (FISH) alone and in combination with the direct viable count method (DVC) to determine if plate counts underestimated total and viable *Salmonella* abundances and if the *Salmonella* cell viability changed after passing through the treatment systems. In most cases, total and viable cell abundances in treatment system effluents were several orders of magnitude higher than cultured cell abundances. Our results indicate that the culture-based method underestimated viable concentrations of the model pathogen, *S. choleraesuis*. *Salmonella* cell viability decreased in effluents during the summer but increased during the winter. Using a culture-based enumeration method alone to determine removal efficiencies of bacterial indicators and pathogens for wastewater treatment systems may result in artificially high estimates of effective treatment.

**Key words** | wastewater treatment, VBNC, FISH, DVC, *Salmonella choleraesuis*

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### INTRODUCTION

Alternative on-site wastewater treatment technologies (e.g. sand filters, peat filters and constructed wetlands) may help resolve problems associated with failing conventional treatment systems at certain sites with restricted area or inadequate soils or soil depth. These alternative treatment systems are needed to mitigate a major source of non-point-source pollution in freshwater and coastal marine zones. Accurate assessment of the potential risks of these technologies requires quantification of the viability of pathogenic cells in them as well as pathogen removal efficiencies. This paper summarizes the results of large-scale field and laboratory experiments utilizing different assays

for determining the viability of a known enteric pathogen, *Salmonella*. Culture-based detection methods to determine removal efficiencies of bacterial indicators and pathogens for wastewater systems may result in artificially high estimates of treatment (Bitton 1999). This paper uses a unique combination of methods, fluorescent *in situ* hybridization (FISH) and the direct viable count (DVC) method, to determine the physiological character of *Salmonella* cells as they pass through these alternative wastewater treatment systems. Our research helps address the question of whether culture techniques alone should be used to assess the bacteriological safety of a water source.

The conventional method of determining viable bacterial cells has been colony counts on culture media. In more recent years, the plate count method has been proven to be occasionally unreliable for the accurate enumeration of enteric bacteria in some aquatic environments (Wagner *et al.* 1993) and evidence indicates that some bacteria may survive for long periods in a viable but non-culturable state (e.g. Chmielewski & Frank 1995; Ravel *et al.* 1995; Roszak & Colwell 1987; Wai *et al.* 1996). Microorganisms may be viable (i.e. capable of growth and dividing) but not form colonies when placed on an appropriate growth medium. Conventional plate counts may be several orders of magnitude lower than viable counts determined by other measures (Bianchi & Giuliano 1996). Inhibitory agents in selective media can prohibit the growth of stressed or sublethally injured target microorganisms, which may otherwise be culturable in non-selective media (Moriñigo *et al.* 1990) or deemed viable by other detection methods (Kogure *et al.* 1978).

Advancements in the application of nucleic-acid-based methods for the detection and identification of microorganisms have expanded our understanding of their distribution, abundance and ecology. Instead of isolating microorganisms from their natural environment and culturing in the laboratory, it is possible to employ other enumeration methods (e.g. FISH, flow cytometry and confocal laser microscopy) to determine bacterial abundances from samples collected directly from the environment (Amann *et al.* 1995; Lopez-Amorós *et al.* 1997). Methods like these are of particular interest when attempting to quantify levels of potentially harmful microorganisms, such as enteric pathogens, in ground and surface waters. Microscopic labeling methods can be used for a variety of environmental samples, including wastewater samples, to detect and compare the removal of enteric pathogens that may otherwise be undetectable through standard plating methods (Wagner *et al.* 1993).

The objective of this study was to determine how alternative on-site wastewater treatment affects the viability and culturability of *Salmonella choleraesuis* (serotype *typhimurium*). We chose this model pathogen because it is a mild pathogen and a nucleic acid probe has been developed to identify this genus using FISH (see Nietupski *et al.* 1992). The goal was to determine whether *Salmonella choleraesuis*

abundances after wastewater treatment were underestimated by plate counts because this pathogen progressed to a viable but non-culturable state in alternative wastewater treatment systems. We realize that our data set is somewhat limited; however, this was a necessary tradeoff in order to be able to conduct field-scale experiments comparing three treatment systems in summer and winter. The results presented here represent an extension of the basic removal determinations for culturable *Salmonella* previously reported in Pundsack *et al.* (2001).

## MATERIALS AND METHODS

Three different duplicated treatment systems were constructed to compare their ability to remove seeded *Salmonella choleraesuis* cells. Alternative on-site systems were constructed between 1995 and 1996 at the Northeast Regional Correction Center (NERCC), a correctional facility for about 125 inmates, approximately 11 miles northeast of Duluth, MN. These systems included subsurface flow constructed wetlands (CWs), intermittent sand filters (SFs) and intermittent peat filters (PFs). Single-pass peat and sand filters and subsurface flow constructed wetlands were the first treatment technologies selected for intensive study in 1994 at the NERCC site based on a comprehensive review of their application in the US and Europe by the authors, regulatory agency staff and several consultants. Key criteria included their expected cost, expected removal efficiency for organic matter and nitrogen and the anticipated level of long-term maintenance in the severe Minnesota winters (see Axler *et al.* (2001) and McCarthy *et al.* (1998) for details). The constructed wetlands (duplicates CW 1 and CW 2) each consisted of two lined 37 m<sup>2</sup> cells in series, filled with gravel to a depth of 0.45 m. The upper cells were planted with local cattails (*Typha latifolia* and *T. angustifolia*) and designed to achieve secondary effluent standards (25 mg TSS L<sup>-1</sup>, 30 mg BOD<sub>5</sub> L<sup>-1</sup> and fecal coliforms to 200 colony forming units (CFU) per 100 mL<sup>-1</sup>) with a nominal hydraulic retention time (HRT) of 6.5 days. The lower cells (planted with greenhouse-raised softstem bulrush, *Scirpus taebermontani*) were intended to enhance nutrient removal by providing additional retention time (total HRT of 13 days).

The surface area of each lined sand filter (duplicates SFW and SFE) was 30 m<sup>2</sup> (5.8 m length by 5.2 m width) with a total bed depth of 1.2 m. The design hydraulic loading rate was 3.15 cm d<sup>-1</sup>. Each peat filter (duplicates PFW and PFE) was 28.7 m<sup>2</sup> (7.0 m length by 4.1 m width), with a total bed depth of 1.4 m. Experiments were conducted in summer 1998 and in winter 1998–99 to determine seasonal effects. Further details about the treatment systems and their seasonal performance for removing the model pathogen, *Salmonella choleraesuis*, may be found in Pundsack (2000) and Pundsack *et al.* (2001). Details regarding the overall design, construction, operation and performance of the treatment systems may be found in McCarthy *et al.* (1997, 1998, 1999) and Axler *et al.* (1999, 2001).

### Culture preparation and inoculation of treatment systems

Batch cultures of streptomycin-resistant *Salmonella choleraesuis* (serotype *typhimurium*; ATCC 23567) were grown in 10 L of nutrient broth (DIFCO 1984) in 5-gallon Pyrex glass carboys. These cultures were grown for 21–36 h at room temperature (19.5–24.5 °C) to achieve concentrations of ~10<sup>8</sup> CFUs mL<sup>-1</sup>.

In separate experiments, the influent to each alternative wastewater treatment system was spiked with *S. choleraesuis* cultures. The systems were amended semi-continuously (i.e. one 10 L batch culture per day for 5–7 consecutive days) during scheduled periods of wastewater loading. Precautions were taken for all systems to prevent back-siphoning to the septic (dosing) tank. Ambient (background) *Salmonella* levels of both the raw wastewater influent and the treated effluents were determined prior to spiking the treatment systems with *S. choleraesuis*.

We have worked in close collaboration with regulatory and wastewater agencies (St. Louis Health Department, Minnesota Pollution Control Agency and Western Lake Superior Sanitary District) since the inception of the NERCC alternative wastewater treatment project to develop appropriate objectives and to select the best sites for research and demonstration projects (see <http://www.bae.umn.edu/~septic/> for the complete project background). As a result, a number of precautions were taken to ensure that the release of cultured *Salmonella choleraesuis* (serotype *typhimurium*) to the test systems would not

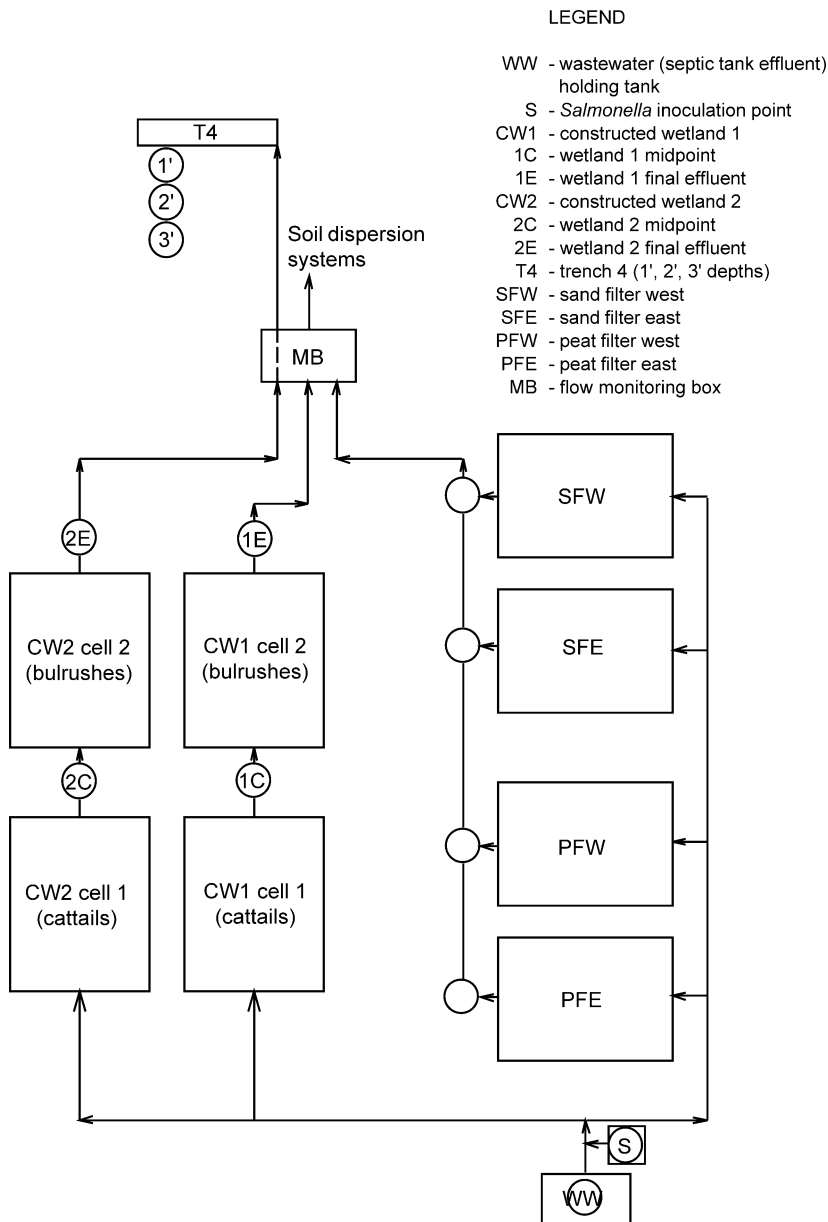
pose a significant health risk to humans or wildlife. The organism itself is a relatively mild enteric pathogen that is not a genetically engineered organism (it is naturally resistant to Streptomycin) and for which the University of Minnesota Institutional Biosafety Committee considers Biosafety Level 2 as appropriate. Typically about 1% of the US population is excreting the organism at any one time (Sterrit & Lester 1988) and *Salmonella typhimurium* is the most common *Salmonella* serotype found in humans suffering from gastroenteritis (McCormick *et al.* 1993). Our background concentration of *Salmonella* measured prior to the seeding experiments were of the order of 10<sup>5</sup> to 10<sup>7</sup> CFUs mL<sup>-1</sup> (see Figures 2–4). Further, there was never any surface exposure of any wastewater and all systems are lined with 40 mil HDPE landfill liners to prevent seepage of partially treated wastewater, and the treated effluent from the alternative treatment systems is then discharged to *conventional* drainfield trenches operated by the correctional facility that have >3 feet of unsaturated soil year-round as prescribed by Minnesota Code 7080 (MPCA 1994). Both our research project's final dispersal site, as well as the correctional facility's drainfields, exceed Minnesota's guidelines for depth of unsaturated soil and are also far removed from any drinking water wells (>250 m). All roads into the test site enter via the correctional facility entrance and are posted with restriction signs and correctional facility security staff patrol these roads. The distance from the test site to the nearest public access road is ~400 m and the nearest private wells are ~1000 m distant. In addition, the test site was selected because at least 5 additional feet of unsaturated soil is located beneath the deepest sampling lysimeters (3 feet deep; McCarthy *et al.* 1997), far exceeding the requirement of only 3 feet of unsaturated soil for untreated septic tank effluent (Minnesota Code 7080). A conservative estimate of the density of spiked *Salmonella* to reach the water table at 8 foot depth was only 0.1 CFUs over the duration of the *poorest* removal experiment, the constructed wetland in winter (using the viability and removal data from this paper and from Axler *et al.* (2001)).

### Sample collection and analysis

Triplicate influent and effluent samples for all treatment systems were collected in sterile 50 mL screw-cap centrifuge

tubes (Figure 1). Influent samples were collected by taking grab samples from the wastewater influent tank. Effluent samples were taken at the outflow discharge point of each treatment system. Outflows were monitored until *Salmonella* concentrations reached background levels and remained constant. Additional samples between cells 1 and

2 of the wetlands (midpoint of the overall system) were taken using a peristaltic pump. All samples were held at 5°C in the laboratory for less than 4 h until further analysis. Influent (septic tank effluent) and treatment system effluents were assayed for total bacterial abundance, total *Salmonella*, viable *Salmonella* and culturable *Salmonella*.



**Figure 1** | Layout of Northeast Regional Correction Center (NERCC) research and demonstration site for comparing year-round performance of alternative on-site wastewater treatment systems. Replicated test systems included subsurface flow constructed wetlands (CWs), intermittent sand filters (SFs), intermittent peat filters (PFs) and conventional drainfield trenches. Wetland treatment systems (CW 1 and CW 2) consisted of two cells in series, the first cells vegetated with cattail (*Typha latifolia* and *T. angustifolia*) and the second cells with bulrush (*Scirpus taebermontani*). Systems were inoculated with *Salmonella choleraesuis* (serotype typhimurium), a model bacterial pathogen, to determine seasonal removal efficiencies. Wetland treatment systems (CW 1 and CW 2) consisted of two cells in series, the first cells vegetated with cattail (*Typha latifolia* and *T. angustifolia*) and the second cells with bulrush (*Scirpus taebermontani*). Systems were inoculated with *Salmonella choleraesuis* (serotype typhimurium), a model bacterial pathogen, to determine seasonal removal efficiencies. Circles represent sampling points. The diagram is not to scale and certain aspects are simplified for the sake of illustration.

The likelihood that culturable *Salmonella* detected in treatment system effluents came from the seeded *Salmonella* cultures was increased because the particular strain of *Salmonella* used to inoculate these systems was streptomycin-resistant. Further measures were required to differentiate between viable *Salmonella* originating from the *Salmonella* culture and from the raw wastewater influent, since the P1200R oligonucleotide probe was only genus-specific.

### Cell fixation and direct counting of total bacterial abundance

For cell fixation, 10 mL of each sample was put in a sterile 15 mL centrifuge tube with 500  $\mu\text{L}$  of 37% formaldehyde and stored at 5 °C. Subsamples of fixed cells were stained with DAPI (38.5  $\mu\text{g mL}^{-1}$ ; concentration of staining solution) for 15–20 minutes and then filtered onto black polycarbonate membrane filters (25 mm diameter; Poretics Corp.). Filters were mounted on pre-cleaned glass microscope slides in immersion oil and a count of total bacteria was determined using epifluorescence microscopy (Porter & Feig 1980).

### Total and viable *Salmonella*

FISH counts will be referred to as “total *Salmonella*” abundances in this paper. Total *Salmonella* abundances were determined in the bacterial community present in the wastewater treatment system samples by epifluorescence microscopy. *Salmonella* cells were hybridized with a Cy3-labeled oligonucleotide probe (P1200R; Integrated DNA Technologies, Inc., Coralville, IA) specific for this genus’ 23S rRNA (Hicks *et al.* 1992; DeLong 1993; Amann *et al.* 1995; Pommepuy *et al.* 1996).

### Cell fixation

For total *Salmonella* counts, subsamples were fixed with 8% paraformaldehyde (in phosphate buffered saline) in a 1:1 ratio (sample to fixative) for 2–24 h at 5 °C. The 8% paraformaldehyde solution was prepared fresh daily. For direct viable *Salmonella* counts, a mixture of nalidixic acid and yeast extract (0.002% w/v and 0.025% w/v,

respectively) was added to unfixed samples (50  $\mu\text{L mL}^{-1}$  final concentration), which were then incubated at 37 °C for 6 h (Kogure *et al.* 1978). Nalidixic acid was added to inhibit DNA synthesis and cell division and yeast extract to provide a nutrient boost for viable cells to elongate. After the incubation period, cells were fixed with 8% paraformaldehyde and processed in the same manner as total *Salmonella* samples. The nalidixic acid method detects synthetically active cells (i.e. cells that are growing) and these *Salmonella* cell counts will be referred to as “viable *Salmonella*” abundances in this paper.

### Cell immobilization

In preparation for cell hybridization, subsamples of fixed cells were stained with DAPI (38.5  $\mu\text{g mL}^{-1}$ ) for 15–20 minutes and filtered onto 0.2  $\mu\text{m}$  pore size black polycarbonate membrane filters. Filters were then immobilized on teflon-coated, gelatin subbed (0.1% gelatin and 0.1%  $\text{CrK}(\text{SO}_4)_2$ ), six-well microscope slides. Two filters (samples) were placed sample side down on each slide with each filter covering two wells. After the filters air-dried, they were gently removed, leaving the fixed cells on the slide. Each slide was then dipped into Milli-Q water for a rinse, followed by dipping serially in 50%, 80% and 95% ethanol rinses for 3 min each. Slides were air-dried in subdued light and stored in a slide box at 5 °C for up to 3 days if they were not hybridized immediately. For every four sample slides, we prepared a slide with positive (i.e. *S. choleraesuis*) and negative (i.e. *Escherichia coli*; ATCC 25922) controls to test the specificity of the probe.

### Hybridization

Hybridization buffer (5  $\times$  SET (0.75 M NaCl, 5 mM EDTA, 0.1 M Tris-HCl [pH 8] / 0.1% sodium dodecyl sulfate) was prepared fresh and equilibrated to 45 °C. Hybridization chambers were prepared by placing a double-folded Kimwipe, soaked with 0.5 mL hybridization buffer (no polyadenylic acid (poly A)), in a 50 mL centrifuge tube. These chambers were equilibrated at 45 °C in a Hybaid oven. Six  $\mu\text{L}$  of hybridization buffer + poly A (0.01%) was added to each well and the slides were then prehybridized

in chambers without added probe for 30 min at 45 °C. After prehybridization, 1 µL (50 ng) of Cy3-labeled P1200R probe (20) was added to one well for each sample and the slides were hybridized overnight at 45 °C. The well that did not receive a probe (blank well) was used to enumerate autofluorescing cells. Coplin jars were filled with wash solution (0.2 × SET) and maintained at 37 °C in a water bath. Slides were washed 3 times for 10 min in this wash solution and allowed to air-dry in subdued light (Stahl & Amann 1991; Hicks *et al.* 1992; Amann *et al.* 1995).

### Total and viable *Salmonella* counts

Both total cells (DAPI stained) and *Salmonella* cells (P1200R probed) were counted in each slide well. Two excitation and emission filter sets were used. The first filter set (Zeiss G365, FT395 and LP420) was used to observe the DAPI stain. The second filter set (Zeiss BP546/12, FL580 and LP590) excited the Cy3-labeled oligonucleotide probe and was used to detect intact *Salmonella* cells. For the direct viable count (DVC) method, *Salmonella* cells were recorded as viable if they were ≥2.5 times longer than an unenriched *Salmonella* cell (unenriched *Salmonella* cells averaged 2–3 µm). At least 10 fields of view (containing at least 300 bacterial cells in total) were counted in each well to determine the number of total cells (DAPI stained) and the number of cells fluorescing in the Cy3 emission region with or without hybridization (Hicks *et al.* 1992).

The ratio of *Salmonella* cells (P1200R probed) to total cells (DAPI stained) was used to calculate concentrations of total and viable *Salmonella*. This approach was used because hybridization slides were prepared using a filter transfer and we could not directly determine cell abundances without knowing the transfer efficiency of microorganisms from membrane filters to gelatin-subbed microscope slides. Instead, it was assumed that *Salmonella* cells transferred equally as well as other cells that were captured on each polycarbonate filter. If this assumption is true, then:

$$\begin{aligned} \text{total } Salmonella \text{ (cells mL}^{-1}\text{)} \\ = \left[ \left( \frac{\text{No. } Salmonella \text{ cells}}{\text{No. total bacteria}} \right) \times \text{direct DAPI count} \right] \end{aligned}$$

where:(No. *Salmonella* cells/No. total bacteria) represents the ratio of Cy3-stained cells (*Salmonella*) to DAPI-stained

cells (total bacteria) observed after filter transfer to hybridized, probed slides; and direct DAPI count = count of total bacterial abundance (cells mL<sup>-1</sup>) determined by direct DAPI counting; and

$$\begin{aligned} \text{viable } Salmonella \text{ (cells mL}^{-1}\text{)} \\ = \left[ \left( \frac{\text{DVC elongated } Salmonella}{\text{DVC total } Salmonella} \right) \times \text{total } Salmonella \right] \end{aligned}$$

where:(DVC elongated *Salmonella*/DVC total *Salmonella*) = proportion of *Salmonella* cells that were ≥2.5 times longer than typical *Salmonella* cells; and total *Salmonella* = the concentration (cells mL<sup>-1</sup>) of total *Salmonella* cells in hybridized samples, calculated using the equation shown above.

### Culturable *Salmonella*

Concentrations of culturable *Salmonella* were determined using a pour plate technique. Bismuth sulfite agar (BSA; Difco) was used to isolate *Salmonella* spp. in samples. Streptomycin-amended (1 µg streptomycin mL<sup>-1</sup>) BSA was used to selectively plate *S. choleraesuis* (ATCC 23567) in serial dilutions of each sample. Pour plates were incubated at 37 °C for 24 h before counting colonies (CFUs). The detection limit for the BSA assay was 0.2–1.3 *Salmonella* cells mL<sup>-1</sup>. Differences between culturable and viable *Salmonella* counts were used to determine whether seeded *Salmonella* cells progressed to a viable but non-culturable state after passing through the wastewater treatment systems.

### Data analysis

Paired *t*-tests were performed to determine whether culturable and viable *Salmonella* counts were significantly different. Only sample dates where both culturable and viable *Salmonella* were observed were used, since the detection limit for viable *Salmonella* was considerably higher (i.e. usually poorer by 2–3 orders of magnitude) than for culturable *Salmonella*. To determine whether cell division occurred in samples with added nalidixic acid, paired *t*-tests were used to compare the number of total *Salmonella* cells counted on total *Salmonella* and DVC slides. No difference in numbers (*p* > 0.05) indicated that

the nalidixic acid concentration was sufficient to prevent cell division.

The proportion of viable *Salmonella* cells that were recovered after passage through each treatment system was compared to that observed in the *Salmonella* inocula and raw wastewater influent. *t*-tests were performed to compare the percent viable *Salmonella* pre- and post-treatment to determine whether the physiological character of *Salmonella* cells changed after passage through the treatment systems.

## RESULTS

To determine the percent contribution of viable *Salmonella* from raw wastewater relative to the *Salmonella* culture inocula, we first determined the average concentration of viable *Salmonella* (cells mL<sup>-1</sup>) in the raw wastewater. This concentration was multiplied by the number of days between the first spike date and the last date that *Salmonella* was detected in the treatment system raw wastewater influent and the result was subsequently multiplied by the average inflow (liters per day) for those dates. The final result estimated the total number of viable *Salmonella* cells entering a treatment system from the raw wastewater alone. The total number of viable *Salmonella* cells in the raw wastewater influent was compared to the total number of viable cells from a *Salmonella* inoculum to yield an estimate of the percent contribution of viable *Salmonella* from raw wastewater. This value ranged from less than 1% for the sand and peat filters to as high as 67% for the constructed wetlands. Between 5–20% of the *Salmonella* cells in the inoculum were viable.

### Summer

For summer wetland experiments, total *Salmonella* concentrations for wetland treatment system effluents averaged  $2-7 \times 10^6$  cells mL<sup>-1</sup> (Figure 2). Total *Salmonella* concentrations were always less than  $2.5 \times 10^4$  cells mL<sup>-1</sup> for sand filter effluents and below the detection limit for peat filter effluents (i.e.  $<3.2-4.2 \times 10^3$  total *Salmonella* cells mL<sup>-1</sup>; Figure 3).

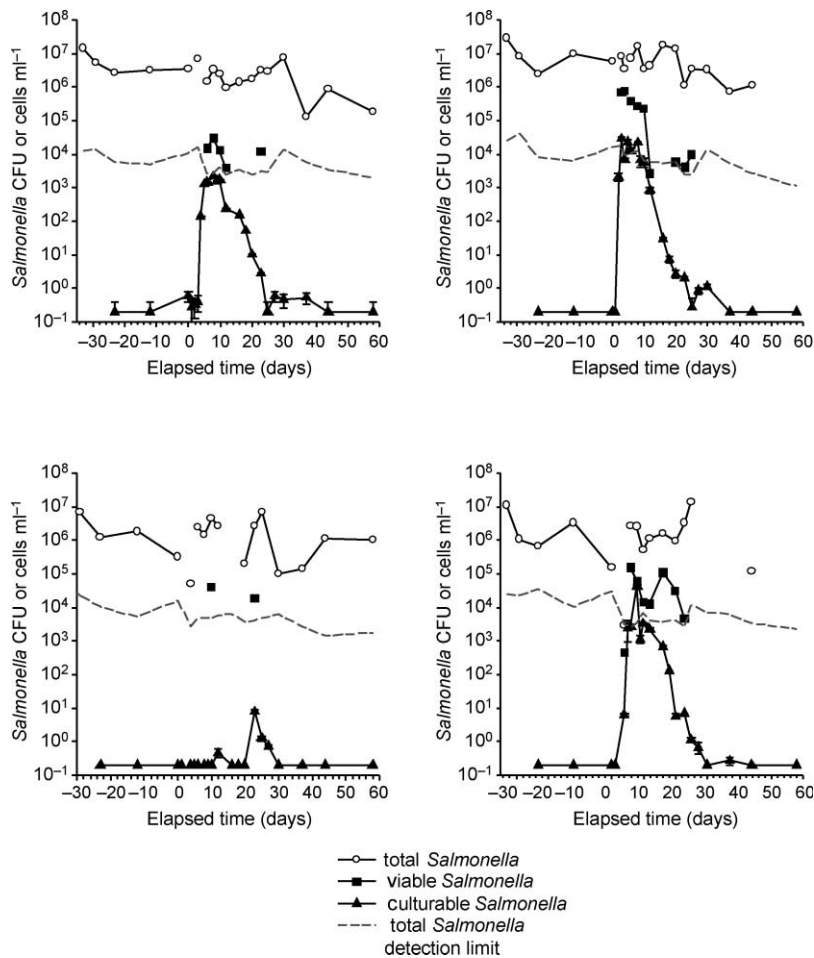
Viable *Salmonella* counts were greater than culturable *Salmonella* counts for the wetland 1 upper cell and both

cells in wetland 2 (all *p*'s < 0.05). Significant differences between percentages of viable *Salmonella* in the raw wastewater influent and wetland 1 effluents could not be determined, as viable *Salmonella* were detected in only one raw wastewater sample. However, a lower percentage of viable *Salmonella* was detected in wetland 1 samples (both upper and lower cells) than in the *Salmonella* inoculum (both *p*'s < 0.05; Table 1, Figure 2). There was no difference between percent viable *Salmonella* in the inocula and treatment system effluents for wetland 2 (Table 1). Wastewater flow into wetland 1 was lower than wetland 2's inflow (Table 1) and it is likely that the reduced flows (increased hydraulic retention time) in wetland 1 were associated with a greater percent loss of *Salmonella* viability since this phenomenon was not observed in the higher flow wetland 2.

There were too few samples in which both culturable and viable *Salmonella* were detected during the summer sand and peat filter experiments to determine if viable and culturable cells abundances were different (Figure 3). Low total *Salmonella* concentrations (i.e. near or below the detection limit) in the sand and peat filter effluents decreased the likelihood that viable *Salmonella* could be observed. The statistical significance of the difference between percent viable *Salmonella* observed in the treatment system inocula (i.e. *Salmonella* inoculum) and sand filter west effluent for the summer experiment could not be determined because only one effluent sample contained viable *Salmonella* cells (*p* > 0.05, Table 1). No viable *Salmonella* were detected in peat filter treatment system effluents or in sand filter east effluent during summer experiments. The replicated sand and peat filters, designated sand filters east and west, and peat filters east and west behaved virtually identically in terms of the relative numbers of *Salmonella* cells that made it through each replicated system (results from summer 1998 and winter 1998–99 sand filter east and peat filter east experiments are shown in Figure 3).

### Winter

For winter experiments, total *Salmonella* concentrations averaged  $1-3 \times 10^6$  cells mL<sup>-1</sup> for wetland treatment system effluents (Figure 4) and  $8 \times 10^4$  to  $2 \times 10^5$  cells



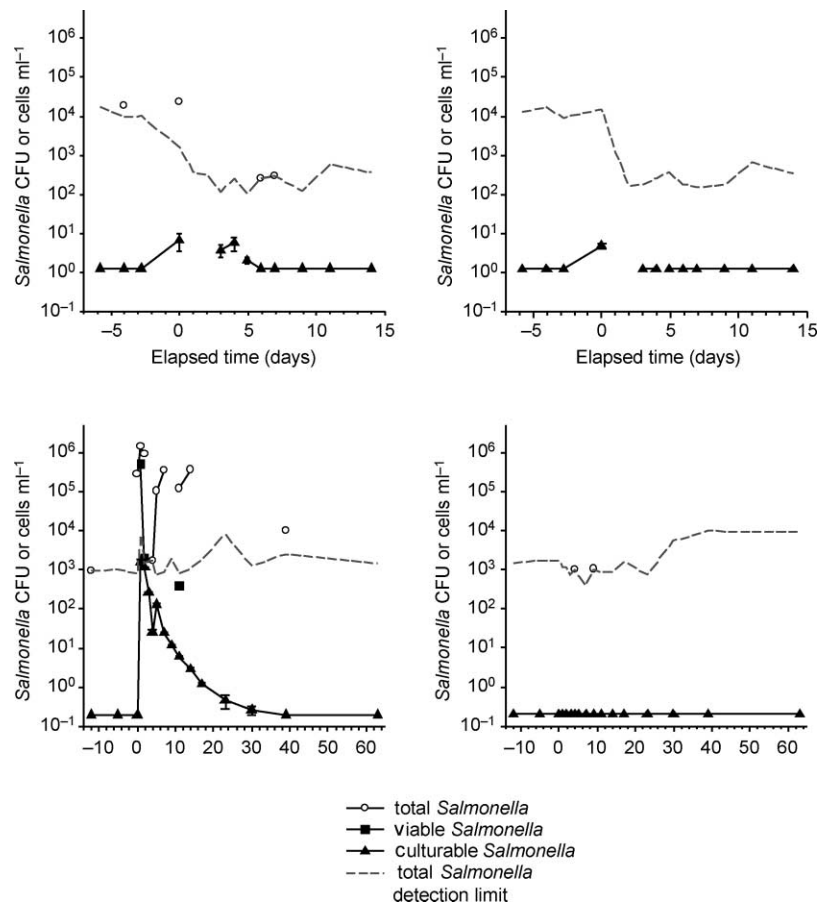
**Figure 2** | Concentrations of total, viable and culturable *Salmonella* in constructed wetland 1 & 2 (inflows = 598Ld<sup>-1</sup> and 882Ld<sup>-1</sup>, respectively) following inoculation with *S. choleraesuis* during the summer 1998 experiment. Upper cell denotes midpoint samples along the length of the treatment systems; lower cell denotes final effluents. Day 0 represents the first inoculation time, and wetlands were inoculated once per day for 7 consecutive days. Error bars for culturable *Salmonella* represent the standard error of three determinations.

mL<sup>-1</sup> for sand filter effluents (Figure 3). Total *Salmonella* concentrations were generally near or below the detection limit in peat filter effluents (i.e.  $\leq 4 \times 10^2$  to  $10^4$  total *Salmonella* cells mL<sup>-1</sup>; Figure 3).

When both viable and total *Salmonella* were detected during the winter wetland experiments, viable *Salmonella* counts averaged approximately two orders of magnitude higher than culturable *Salmonella* counts (Figure 4). This difference was only significant ( $p < 0.05$ ;  $n = 14$ ) in the upper cell of wetland 2. For winter peat filter experiments there were too few samples in which both culturable and viable *Salmonella* were detected to determine whether they were significantly different (Figure 3). A higher percentage of viable *Salmonella* was detected in wetland 1 effluents

(both upper and lower cells) than in the *Salmonella* inocula (both  $p$ 's  $< 0.05$ ; Table 2). The effluent from the lower cell of wetland 1 had a greater percentage of viable *Salmonella* than the raw wastewater influent ( $p < 0.05$ ), while the upper cell effluent did not ( $p > 0.05$ ). There was no difference between percent viable *Salmonella* observed in the treatment system inocula (i.e. *Salmonella* culture and raw wastewater) and in wetland 2 effluent samples (both upper and lower cells,  $p > 0.05$ ; Table 2). Total *Salmonella* concentrations were always less than  $10^4$  cells mL<sup>-1</sup> ( $n = 2$ ) for the peat filters, which made it unlikely that viable *Salmonella* would be detected since these values were near the detection limit (Figure 3). During the winter, viable and culturable *Salmonella* concentrations were not different in





**Figure 3** | Concentrations of total, viable and culturable *Salmonella* in sand filter east and peat filter east following inoculation with *S. choleraesuis* during the summer 1998 and winter 1998–99 experiments. Day 0 represents the first inoculation time, and sand and peat filters were inoculated once per day for 5 consecutive days. Error bars for culturable *Salmonella* represent the standard error of three determinations.

the sand filter effluents (Figure 3; both  $p$ 's > 0.05). There was no difference between percent viable *Salmonella* in the treatment system inocula and the treatment system effluents for sand and peat filters ( $p$ 's > 0.05; Table 2).

## DISCUSSION

Culture-based and nucleic acid-based methods for detecting microorganisms in aquatic environments each have certain advantages and disadvantages. A drawback of culture-based methods is that *Salmonella* which are sublethally injured or starved, yet viable, may not form colonies when incubated in the appropriate growth medium (Pommepuy *et al.* 1996).

Ribosomal RNA-based methods can detect non-viable as well as viable microorganisms and thus it is usually

necessary to employ a combination of techniques to determine the physiological state of pathogens such as *Salmonella* (Tolker-Nielsen *et al.* 1997). In our experiments, the P1200R probe was used to determine total *Salmonella*. The same probe in conjunction with the DVC technique was used to estimate the number of viable *Salmonella* cells (i.e. substrate responsive). The detection limits for total *Salmonella* averaged 10<sup>3</sup> to 10<sup>4</sup> cells mL<sup>-1</sup>, or 2–3 orders of magnitude higher (i.e. poorer) than the detection limit for culturable *Salmonella*. The high concentration of native wastewater microorganisms in the septic tank (~10<sup>7</sup> to 10<sup>9</sup> cells mL<sup>-1</sup>) and treatment system effluents (~10<sup>5</sup> to 10<sup>8</sup> cells mL<sup>-1</sup>) made quantification of *Salmonella* cells challenging since total bacterial abundance was often considerably greater than the abundance of the model pathogen, *Salmonella choleraesuis*. We have found that having too

**Table 1** | Proportion of viable versus total *Salmonella* cells following passage through alternative on-site wastewater treatment systems for summer 1998 experiments. The treatment system inocula were batch cultures of *Salmonella* and indigenous *Salmonella* in the raw wastewater. The total number of viable *Salmonella* cells in the raw wastewater influent was compared to the total number of viable *Salmonella* cells contributed by the *Salmonella* inocula to give an estimate of the relative percent contribution of viable *Salmonella* from raw wastewater. Numbers in parentheses are the standard error of the mean ( $n = 7$  for constructed wetland inocula and  $n = 5$  for *Salmonella* culture sand and peat filter inocula). Numbers of effluent samples positive for viable *Salmonella* are reported individually for each treatment system. A letter next to an effluent value represents a significant difference ( $p < 0.05$ ) compared to the *Salmonella* inoculum (see notes below table).  $Q$  = septic tank effluent inflow rate. See Figure 1 for sampling points

Treatment system	Treatment system inocula			Treatment system effluent	
	% viable <i>Salmonella</i>			% viable <i>Salmonella</i>	$n$
	<i>Salmonella</i> Inoculum	Raw wastewater	% contribution from raw wastewater		
Constructed Wetland 1 ( $Q = 598 \text{ L d}^{-1}$ )	4.6 (1.3)	28.9	37	Upper Cell 1 (C) 0.7 (0.1) <sup>a</sup>	5
				Lower Cell 2 (E) 0.8 (0.1) <sup>a</sup>	2
Constructed Wetland 2 ( $Q = 882 \text{ L d}^{-1}$ )	4.6 (1.3)	28.9	67	Upper Cell 1 (C) 5.0 (2.4)	9
				Lower Cell 2 (E) 4.7 (1.7)	8
Sand Filter West ( $Q = 1,079 \text{ L d}^{-1}$ )	5.4 (1.3)	0	<1	10.0	1
Sand Filter East ( $Q = 931 \text{ L d}^{-1}$ )	5.3 (0.5)	0	<1	nd <sup>1</sup>	
Peat Filter West ( $Q = 560 \text{ L d}^{-1}$ )	4.7 (0.8)	0	<1	nd <sup>1</sup>	
Peat Filter East <sup>1</sup> ( $Q = 621 \text{ L d}^{-1}$ )	6.0 (1.1)	0	<1	nd <sup>1</sup>	

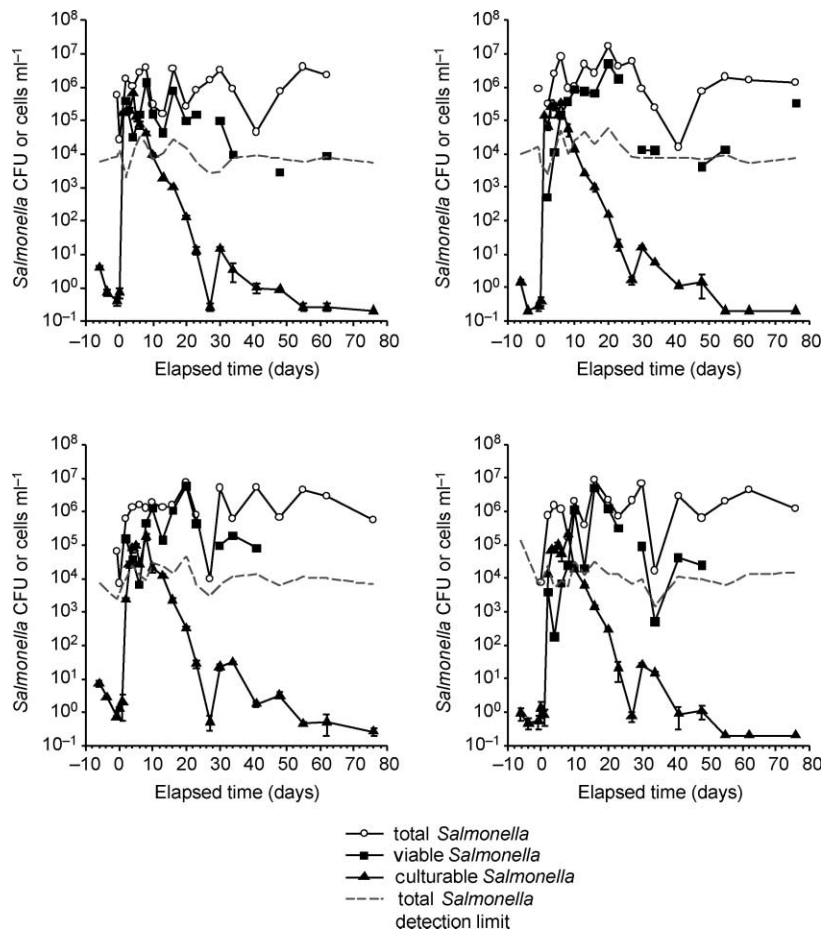
<sup>1</sup>nd = not detected.

<sup>a</sup> = significant difference between *Salmonella* inoculum and treatment system effluent *Salmonella*.

many cells on hybridization slides can result in non-specific binding of probe to non-target microorganisms, as well as increasing difficulties in distinguishing target microorganisms. For our experiments, tests were performed to optimize the concentration of cells during *in situ* hybridizations by filtering appropriate sample volumes (generally 1–2 mL of treatment system effluent).

The relatively low percentage (5–20%) of viable *Salmonella* in the inoculum was probably due to the inoculum cells being harvested after the exponential growth phase. With the exception of the wetland experiments, the raw wastewater influent did not contribute a significant portion of the viable *Salmonella* cells observed in the treatment system influents (Tables 1 and 2). The higher percent contribution of viable *Salmonella* from raw wastewater for the constructed wetlands (29–67%) may be due to slight differences in system plumbing for the wetlands, potentially allowing a degree of backflow from the point of *Salmonella* inoculation into the raw

wastewater holding tank, or to higher numbers of *Salmonella* cells in the wastewater dosing tank during the experimental period (Tables 1 and 2). During the summer and winter, the raw wastewater contribution was estimated to be less than 1% of the viable *Salmonella* observed in the sand and peat filter influents. Thus, viable *Salmonella* cells observed in these treatment system effluents quite likely originated from the *Salmonella* inocula. Unfortunately, there were usually too few effluent samples from these systems that contained enough viable *Salmonella* cells to determine whether the percentage of viable *Salmonella* was different from the *Salmonella* inocula. During winter, >95% of culturable *Salmonella* cells were removed by these systems and >99.999% of these cells were removed during the summer (Pundsack *et al.* 2001). Viable *Salmonella* from the raw wastewater influent could account for ~29–67% of all viable *Salmonella* entering the constructed wetlands during the summer and winter experiments. Usually, the proportion of viable *Salmonella* cells in the



**Figure 4** | Concentrations of total, viable and culturable *Salmonella* in constructed wetland 1 and 2 following inoculation with *S. choleraesuis* during the winter 1998–99 experiment. Upper cell denotes midpoint samples along the length of the treatment systems; lower cell denotes final effluents. Day 0 represents the first inoculation time, and wetlands were inoculated once per day for 5 consecutive days. Error bars for culturable *Salmonella* represent the standard error of three determinations.

wetland effluents was similar to the proportions observed in the raw wastewater influent. In only one case, the winter wetland experiment, was the proportion of viable *Salmonella* in the raw wastewater different (i.e. smaller) than the treatment system effluent (Table 2).

Following *Salmonella* inoculation, concentrations of total *Salmonella* would be expected to be high in the treatment system effluents due to the large influx of *Salmonella* cells from the inoculum. However, high background concentrations of total *Salmonella* were observed in the constructed wetland effluents, especially during the summer (Figure 2). This observation was troubling because, prior to inoculation, high concentrations of total *Salmonella* within the wetland effluents relative to the raw wastewater influents suggest that *Salmonella* cells may

have been multiplying in these wetlands or the wetland treatment systems may retain *Salmonella* cells which are later released in the effluent.

We examined all treatment systems with enough viable *Salmonella* in the effluents to determine whether the physiological character (i.e. percent viable) of the *Salmonella* cells in the effluents was different from the inoculum. Wetland 1 was the only treatment system where the proportion of viable *Salmonella* changed seasonally (Tables 1 and 2). The proportion of viable *Salmonella* in these effluents was smaller compared to the inoculum during the summer experiment, but the proportion of viable cells was larger after passing through the same wetland in the winter. Reduced flows (i.e. increased hydraulic retention time) in wetland 1 may have contributed to the loss of cell

**Table 2** | Proportion of viable versus total *Salmonella* cells following passage through alternative on-site wastewater treatment systems for winter 1998–99 experiments. The treatment system inocula were batch cultures of *Salmonella* and indigenous *Salmonella* in the raw wastewater. The total number of viable *Salmonella* cells in the raw wastewater influent was compared to the total number of viable *Salmonella* cells contributed by the *Salmonella* inocula to give an estimate of the relative percent contribution of viable *Salmonella* from raw wastewater. Numbers in parentheses are the standard error of the mean ( $n = 5$  for *Salmonella* culture treatment system inocula). Numbers of effluent samples positive for viable *Salmonella* are reported individually for each treatment system. A letter next to an effluent value represents a significant difference ( $p < 0.05$ ) compared to the *Salmonella* inoculum (see notes below table).  $Q$  = septic tank effluent inflow rate. See Figure 1 for sampling points

Treatment system	Treatment system inocula			Treatment system effluent	
	% viable <i>Salmonella</i>			% viable <i>Salmonella</i>	$n$
	<i>Salmonella</i> inoculum	Raw wastewater	% contribution from raw wastewater		
Constructed Wetland 1 ( $Q = 545 \text{ L d}^{-1}$ )	7.9 (2.9)	14.2 (5.1)	29	Upper Cell 1 (C) 21.0 (5.1) <sup>a</sup>	11
				Lower Cell 2 (E) 31.8 (8.6) <sup>a,b</sup>	12
Constructed Wetland 2 ( $Q = 708 \text{ L d}^{-1}$ )	7.9 (2.9)	14.2 (5.1)	38	Upper Cell 1 (C) 21.9 (7.4)	11
				Lower Cell 2 (E) 20.4 (7.2)	12
Sand Filter West ( $Q = 912 \text{ L d}^{-1}$ )	20.9 (10.0)	5.5 (0.9)	<1	2.4 (2.0)	3
Sand Filter East ( $Q = 863 \text{ L d}^{-1}$ )	20.9 (10.0)	5.5 (0.9)	<1	12.2 (12.0)	3
Peat Filter West ( $Q = 977 \text{ L d}^{-1}$ )	20.9 (10.0)	5.5 (0.9)	<1	25	1
Peat Filter East <sup>1</sup>					

<sup>1</sup>The peat filter east experiment was terminated early because the supply line to PFE froze on the third day of *Salmonella* inoculation.

<sup>a</sup> = significant difference between *Salmonella* inoculum and treatment system effluent *Salmonella*.

<sup>b</sup> = significant difference between raw wastewater *Salmonella* and treatment system effluent *Salmonella*.

viability during the summer experiment. A similar cell viability change was not observed in wetland 2 where the flow was higher. During the winter, however, the opposite pattern was observed. The lower flow, wetland 1 effluents had larger percentages of viable *Salmonella* than the inoculum. Although flow rate may affect the retention of pathogenic bacteria like *Salmonella* in these wetland systems, it appears that other factors besides the season also determine if cell viability will change in these systems.

The results of this study indicate that a selective, culture-based method underestimated the abundance of viable *Salmonella* cells compared to the DVC method used in combination with FISH. Other culture media for isolating *Salmonella* spp. may have produced slightly different results (Hu & Gibbs 1995; Ruiz *et al.* 1996). Our investigation confirms other researcher's (Roszak *et al.* 1984; Chmielewski & Frank 1995) conclusions that *Salmonella* can enter a viable but non-culturable state where

viable cell abundances measured by the DVC method are higher than plate count measurements. Viable *Salmonella* cells that go undetected by conventional plating procedures may present environmental and public health threats. One key question is whether viable but non-culturable cells which pass through treatment systems like these retain pathogenic and infective characteristics (McCay 1992; Weichart & Kjelleberg 1996). At low cell concentrations, a discrepancy between culturable and viable *Salmonella* counts may not have health risk implications, depending on where the water is discharged after treatment and uses of the impacted water supply. At higher cell concentrations or when large anomalies between these counting methods exist, however, the public health consequences could be very important.

Several authors have questioned basing pathogenicity and public health risk calculations on culture-based methods for enumerating microorganisms (McCay 1992;

Pommepuy *et al.* 1996). Using culture-based enumeration methods alone to determine removal efficiencies of indicator bacteria and pathogens for wastewater treatment systems may result in artificially high estimates of effective treatment (Pundsack 2000; Pundsack *et al.* 2001), the present study included. Thus, when setting treatment standards, risk assessments should be conservative if these standards are based on culture-based enumerations because actual pathogen concentrations may be significantly higher. The viable but non-culturable cell phenomenon presents the problem of whether culture-based techniques alone should be used to assess the bacteriological safety of a water source.

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