Removal of *Salmonella* and microbial indicators in constructed wetlands treating swine wastewater

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**Abstract** Reductions of *Salmonella* bacteria and enteric microbial indicator organisms were measured in swine wastewater treated by a field-scale surface flow (SF) constructed wetland at a commercial hog nursery in North Carolina and in laboratory-scale SF and subsurface flow (SSF) constructed wetland reactors. Overall reductions of *Salmonella*, fecal coliforms and *E. coli* were 96, 98 and 99%, respectively, in the two-cell field-scale wetland. Somatic and F-specific coliphage viral indicators were reduced by 99 and 98%, respectively. Reductions of *Salmonella*, fecal coliforms and *E. coli* were similar in the first cell of the field system and in the laboratory-scale SF wetland operated at a TKN loading of 25 kg ha\(^{-1}\) d\(^{-1}\) and 30°C (approximately 70, 90 and 90%, respectively). In the SSF wetland reactor, *Salmonella* and fecal coliform reductions were 80 and 98%, respectively, at a 40 kg TKN ha\(^{-1}\) d\(^{-1}\) loading and 99.8 and 99.99%, respectively, at a 10 kg TKN ha\(^{-1}\) d\(^{-1}\) loading. These results show that SF constructed wetlands can be effective for reducing enteric pathogens in swine wastewater and that greater removals can be achieved using SSF designs and lower TKN loading rates.

**Keywords** Constructed wetlands; pathogens; salmonella; swine waste; wastewater treatment

**Introduction** Manure and wastewater from animal feeding operations (AFOs) are potential sources of a wide range of pollutants, including pathogens, that can be transported to environmental resources near farms. Removal or inactivation of enteric pathogens in swine wastewater is important because potentially infectious human pathogens (e.g., *Salmonella, Yersinia, Cryptosporidium parvum*, emerging viruses like swine hepatitis E virus) may be present and transported to nearby water resources by surface water runoff and groundwater infiltration (Cole *et al.*, 1999). Fecal microbes have been found at high concentrations in flushed swine waste (Hill and Sobsey, 1998). In the US, waste generated on swine farms (as well as dairy farms) is often stored in lagoons from which the liquid is periodically applied to land application fields (i.e., sprayfields). Public pressure has been increasing to develop alternative treatment systems to lagoons due to concerns regarding potential public health and environmental risks of lagoon-sprayfield waste management systems. Constructed wetlands represent a promising alternative or additional treatment system for wastewaters generated by animal feeding operations (AFOs). Over 100 constructed wetland systems are treating livestock wastewater in the US (CH2M Hill and Payne Engineering, 1997). Most of these are surface flow (SF) systems, but some use subsurface flow (SSF) designs.

Previous research indicates that enteric microbe removal efficiency in constructed wetlands can be affected by changes in hydraulic loading rate (HLR) and resultant hydraulic residence time (HRT) (Tanner *et al.*, 1995), the presence of vegetation (Soto *et al.*, 1999), and whether the systems are SF or SSF (Kadlec and Knight, 1996). Enteric bacteria have been reported to be removed by 90–99.9% (Tanner *et al.*, 1995; Ottová *et al.*, 1997; Gerba *et al.*, 1999) and viruses by 90–99% (Gersberg *et al.*, 1989; Gerba *et al.*, 1999) in SF and SSF constructed wetlands. Although the potential for substantial reductions of enteric microbes in constructed wetlands is suggested by previous research, much of this research has been...
limited to the analysis of fecal coliforms and other bacterial indicators which may not be indicative of the removal of other microbes, such as viruses, protozoan parasites or helminths. Additionally, little research has focused on the removal of frank pathogens, such as *Salmonella*, in constructed wetlands. Available data indicate that protozoan pathogens such as *Cryptosporidium parvum* and *Giardia lamblia* may be less effectively removed than enteric bacteria and viruses in wetland systems, with reported removal efficiencies of less than 90% (Gerba *et al.*, 1999). Research on the removal of free-living amoeboae indicates that these microorganisms can be removed by 75-95% in vegetated SSF wetlands (Rivera *et al.*, 1995). Other research indicates that helminth ova such as *Ascaris lumbricoides* can be removed by 80-90% in SSF wetlands (Stott *et al.*, 1997). The spore-forming bacterium, *Clostridium perfringens*, was investigated in this study as an indicator for the removal of environmentally stable protozoan and helminth parasites like *C. parvum* and *Ascaris* spp., respectively.

The objectives of this study were to (1) quantify reductions of the pathogen, *Salmonella*, and enteric bacterial and viral indicator microbes in a two-cell SF wetland operating at a commercial swine farm and in laboratory-scale SF and SSF wetland reactors, (2) investigate the effect of loading rate on enteric microbe reductions in the laboratory-scale wetland reactors, (3) evaluate the effect of vegetation on enteric microbe removals by comparing removals in vegetated and non-vegetated SSF reactors, and (4) investigate correlations between microbial indicator and *Salmonella* reductions in constructed wetlands.

**Methods and materials**

**Operation and sampling of constructed wetland systems**

The field-scale constructed wetland system was installed at a 2,600-head swine nursery in North Carolina, USA in 1992 (Hunt *et al.*, 1998). The SF system contained two cells (each 3.6 m × 33.5 m) in series, planted with bur-reed (*Sparganium americanum*) and cattails (*Typha angustifolia* and *Typha latifolia*). Wastewater from the anaerobic lagoon at the farm was diluted 1:1 with water and pumped through the system at a total nitrogen loading rate of 25 kg ha⁻¹ d⁻¹. The hydraulic loading rate (HLR) of lagoon liquid to the field system averaged 2.0 cm d⁻¹.

Three laboratory-scale reactors (76 cm × 30 cm × 61 cm polyethylene tanks) were installed in a walk-in incubator in October 1998: a SF reactor with soft-stem bulrush (*Scoenoplectus validus*) planted in sandy loam (30 cm deep); a SSF reactor with soft-stem bulrush planted in 30-cm-deep expanded-slate gravel (9.5 mm average diameter) (Carolina Stalite Co.; Salisbury, North Carolina); and an non-vegetated 30-cm deep SSF expanded-slate gravel reactor. Full spectrum “Sunshine” plant grow-lights (General Electric) were suspended above each reactor. Beginning in February 1999, lagoon liquid diluted 1:1 with tap water was pumped from a refrigerated central distribution tank into each reactor using peristaltic pumps. The incubator temperature was set at 30°C to model Summer temperature conditions. Between September 1999 and March 2000, the reactors were studied at an initial total Kjeldahl nitrogen (TKN) loading rate of 40 kg ha⁻¹ d⁻¹ (3.8 cm d⁻¹ HLR), an intermediate TKN loading rate of 25 kg ha⁻¹ d⁻¹ (2.3 cm d⁻¹ HLR), and a final TKN loading rate of 10 kg ha⁻¹ d⁻¹ (1.1 cm d⁻¹ HLR). Tracer tests were conducted at each loading rate using sodium fluoride to measure the hydraulic residence time (HRT) in each reactor. *Salmonella typhimurium*, isolated from a commercial hog farm lagoon, was spiked into the influent tank to maintain an approximate influent concentration of 1000 000 MPN per 100 mL.

**Sample collection and analysis**

Grab samples were collected from the field system influent and effluent, as well as between
the two cells. Between March 1997 and May 2000, 18 sets of samples were collected from the field-scale system and analyzed for fecal coliforms, *Eschericia coli*, enterococci, *Clostridium perfringens* spores, somatic coliphages and male-specific (F-specific) coliphages. Samples were also analyzed for *Salmonella* spp., chemical oxygen demand (COD), pH and total suspended solids (TSS) during 9 rounds of sampling conducted between December 1998 and May 2000. For the laboratory study, samples were collected from the influent tank and the effluent from each of the three reactors. At least four rounds of sampling were conducted at each TKN loading rate. Samples were analyzed for fecal coliforms, *E. coli*, enterococci, *C. perfringens* spores, somatic coliphages, F-specific coliphages, *Salmonella* spp., pH, COD, and TSS.

Fecal coliforms and *E. coli* were enumerated by membrane filtration as described in *Standard Methods for the Examination of Water and Wastewater* (1999). Enterococci were enumerated by incubating membrane filters on modified mE agar (Difco) for 48 h at 41°C (Levin et al., 1975). *C. perfringens* spores were analyzed by filtering heat-treated (60–70°C for 20 minutes) samples and incubating membranes on mCP agar (Acumedia®) in an anaerobic jar for 18–24 h at 41°C (Bisson and Cabelli, 1979). Viral indicators were enumerated using the single-agar layer, pour plate plaque technique (Grabow and Coubrough, 1986), with somatic and F-specific coliphages detected using host bacteria *E. coli* CN-13 and *E. coli* Famp, respectively. *Salmonella* were enumerated using the most probable number (MPN) technique as follows: pre-enrichment for 20–24 h at 37°C in buffered peptone water (Difco) (Edel and Kampelmacher, 1973); enrichment for 24 h at 43°C in Rappaport-Vassiliadis R10 broth (Difco) (Vassiliadis, 1983); parallel isolation on Salmonella-Shigella (Difco) and Rambach® agar (CHROMagar Microbiology); and biochemical testing of a subset of presumptive positives using BBL® Enterotube™ II media (Becton Dickinson). COD was measured using the Hach COD System and a Spectronic 1201 spectrophotometer (Milton Roy) set at $\lambda = 620$ nm. TSS was measured using Standard Method 2540 D (1999).

For tracer tests, fluoride ion concentration was measured using an Accumet™ ion-selective electrode. First-order volumetric and areal rate constants ($k$) were calculated using standard exponential decay equations (Kadlec and Knight, 1996).

The nonparametric Wilcoxon Matched Pairs test was used to (1) compare influent and effluent data for individual microbes to determine whether the treatment systems achieved significant reductions of these microbes; (2) evaluate whether differences in measured treatment effectiveness between the various microbes were significant; and (3) investigate differences in treatment effectiveness for the laboratory-scale wetland reactors. Correlation analysis of the log$_{10}$ reductions measured for the different microbes was performed using the Spearman Rank Order nonparametric method. Linear regression analysis was used to determine if loading rate was significantly associated with enteric microbe removals. For all statistical analyses, significance is considered to be a $p$ value $\leq 0.05$. All statistical analysis was performed using *Statistica* software (StatSoft, Inc.).

**Results and discussion**

Diluted lagoon liquid pumped through the SF constructed wetland at the hog nursery had an average COD of 620 mg L$^{-1}$, TSS of 190 mg L$^{-1}$ and pH of 7.9 (Table 1). COD and TSS were reduced by 71 and 92%, respectively, in effluent from the system (Cell 2 effluent).

The influent to the wetland system had geometric mean concentrations of fecal coliforms and *E. coli* of 240 000 and 180 000 colony forming units (CFU) per 100 mL, respectively (Figure 1). These bacterial indicators were significantly reduced in each cell of the wetland system: 1.0 log$_{10}$ (91%) and 1.1 log$_{10}$ (92%), respectively, in Cell 1 and 0.7 log$_{10}$ (80%) and 0.8 log$_{10}$ (84%), respectively, in Cell 2. Overall reductions for fecal coliforms and *E. coli* were 1.7 log$_{10}$ (98%) and 1.9 log$_{10}$ (99%), respectively.
Enterococci were less effectively reduced in the wetland system than were fecal coliforms and \textit{E. coli}, with measured reductions of $0.7 \log_{10}$ (80\%) in Cell 1 and $0.9 \log_{10}$ (87\%) in effluent from the system. Enterococci concentrations in wetland system effluent were significantly lower than in system influent, but the concentrations in Cell 2 effluent were not significantly different than in Cell 1 effluent. Overall, enterococci reductions in the constructed wetland were significantly lower than for all the other enteric microbes studied. The reductions of the other enteric microbes were not significantly different from each other in the wetland system. Enterococci (as well as other fecal streptococci) are generally thought to be more resistant to environmental degradation than fecal coliforms, including \textit{E. coli}. These data suggest that enterococci may be a good indicator for more environmentally stable bacterial pathogens. It is also possible, however, that the low reductions of enterococci during some sampling rounds may reflect the reproduction of these organisms in the wetland system. On three occasions, enterococci concentrations in effluent from the wetland system were higher than in system influent, while fecal coliform and \textit{E. coli} concentrations were reduced by 1.5 to $2 \log_{10}$. Research has shown that enterococci can exist naturally and reproduce on some plant species (Clausen \textit{et al.}, 1977; Anderson \textit{et al.}, 1997).

\textit{Salmonella} were measured at far lower concentrations than the indicator bacteria in influent to the wetland system (Figure 1), although they were readily detectible in 100 mL volumes. \textit{Salmonella} were reduced from an influent geometric mean of 350 MPN/100 mL to a mean of 130 MPN/100 mL in Cell 1 (a $0.4 \log_{10}$, or 63\% reduction) and a mean of 12 MPN/100 mL in effluent from the system (a $1.5 \log_{10}$, or 96\% reduction).

\begin{table}
\centering
\caption{Average wastewater characteristics in surface flow constructed wetland at commercial swine nursery, North Carolina, USA}
\begin{tabular}{lccc}
\hline
Sample Location & COD (mg L$^{-1}$) & TSS (mg L$^{-1}$) & pH \\
\hline
System Influent & 620 & 190 & 7.9 \\
Cell 1 Effluent & 250 & 33 & 7.8 \\
Cell 2 Effluent & 180 & 15 & 7.8 \\
\hline
\end{tabular}
\end{table}

\textbf{Figure 1} Geometric mean concentrations of \textit{Salmonella} and microbial indicators in surface flow constructed wetland treating swine lagoon liquid.
reductions in Cell 1 were not significant \((p = 0.08)\), but were significant in system effluent. *Salmonella* concentrations varied greatly in the wetland system, which may have been due to seasonal and climatic conditions as well as differences in the prevalence of infection and fecal excretion levels in the numerous groups of animals that moved through the nursery facility during the study. Variations in constructed wetland treatment performance for *Salmonella* reduction were significantly correlated with reductions of fecal coliforms \((R = 0.85)\), *E. coli* \((R = 0.82)\) and *C. perfringens* spores \((R = 0.71)\), but were not significantly correlated with reductions in enterococci or the coliphages. *Salmonella* reductions in the SF wetland were not significantly different than fecal coliform or *E. coli* reductions, but were significantly higher than enterococci reductions.

*C. perfringens* spore concentrations were reduced by 1.2 log\(_{10}\) (93%) in Cell 1 effluent and by 1.5 log\(_{10}\) (97%) in system effluent. The 95% confidence limits for *C. perfringens* spore reductions overlap slightly between Cell 1 and Cell 2 effluent. This was likely due to highly variable seasonal and climatic conditions that affected both the constructed wetlands performance as well as the treatment performance of the lagoon that was used as the source of wastewater for the study. When the data were analyzed using paired nonparametric statistics it was determined that the effluent concentrations of *C. perfringens* spores from Cell 2 were significantly lower than corresponding concentrations in influent to the cell. Reductions of *C. perfringens* spores were significantly correlated with reductions of *Salmonella* \((R = 0.71)\), fecal coliforms \((R = 0.64)\), *E. coli* \((R = 0.68)\) and somatic coliphages \((R = 0.59)\). These results suggest that more environmentally-stable enteric microbes (e.g., *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts and helminth ova) may also be significantly removed from wastewater by similarly designed and operated SF constructed wetland systems. Because bacterial spores and parasites are relatively stable in the environment, release of these microbes from the wetland treatment system is a possibility, especially during system perturbations (e.g., precipitation events).

Somatic and F-specific coliphages were reduced to a similar, and significant, extent in each cell of the SF constructed wetlands system: 1.0 log\(_{10}\) (90%) and 0.8 log\(_{10}\) (83%), respectively, in Cell 1 and 0.9 log\(_{10}\) (87%) and 1.0 log\(_{10}\) (90%), respectively, in Cell 2. Overall reductions of these coliphages in the wetland system were 1.9 log\(_{10}\) (99%) and 1.8 log\(_{10}\) (98%), respectively. Log\(_{10}\) reductions of these two viral indicator microbes were more strongly correlated with each other \((R = 0.83)\) than with the other microbes studied. Somatic coliphage reductions were also significantly correlated with fecal coliforms \((R = 0.54)\), *E. coli* \((R = 0.51)\) and somatic coliphages \((R = 0.59)\). Other than with somatic coliphages, F-specific coliphage reductions were only significantly correlated with enterococci \((R = 0.63)\).

The first-order areal rate constants \((k_a)\) for inactivation/removal of enteric microbes in the two-cell SF constructed wetland (as cm/d) were calculated to be: 6.7 for *Salmonella*, 8.0 for fecal coliforms, 8.7 for *E. coli*, 4.0 for enterococci, 8.9 for somatic coliphages, 7.8 for F-specific coliphages, and 7.0 for *C. perfringens* spores. The calculated rate constant for fecal coliforms is within the range of reported first order areal decay rates for municipal SF constructed wetland systems (Kadlec and Knight, 1996).

In the laboratory-scale SF constructed wetland reactor, *S. typhimurium* was reduced by 0.5 log\(_{10}\) (71%) at a TKN loading of 25 kg ha\(^{-1}\) d\(^{-1}\) (Figure 2). This result is similar to the 0.4 log\(_{10}\) reduction measured in Cell 1 of the field-scale SF constructed wetland operated at a total nitrogen loading of 25 kg ha\(^{-1}\) d\(^{-1}\) (50 kg ha\(^{-1}\) d\(^{-1}\) if considering the loading to Cell 1 only). Fecal coliform and *E. coli* reductions in the laboratory-scale SF reactor at a TKN loading of 25 kg ha\(^{-1}\) d\(^{-1}\) (0.9 and 1.0 log\(_{10}\) respectively) were also similar to corresponding reductions in the first cell of the field system (1.0 and 1.1 log\(_{10}\) respectively). In the laboratory-scale SF reactor, treatment effectiveness for the enteric bacteria was lowest at...
the highest loading rates, but the association between loading rate and microbe removal was significant only for fecal coliforms. In the SSF reactors, loading rate was significantly associated with log_{10} reductions of \textit{S. typhimurium}, fecal coliforms and \textit{E. coli}.

\textit{Salmonella}, fecal coliform and \textit{E. coli} reductions were significantly greater in the SSF reactors than in the SF reactor at the same TKN loading rates (Figure 2). Reductions of these microbes decreased in the vegetated SSF reactor as the TKN loading rate was increased: 2.9 log_{10} to 0.7 log_{10} (99.8 to 80\%) for \textit{Salmonella}, 4.0 log_{10} to 1.7 log_{10} (99.99 to 98\%) for fecal coliforms, and 3.9 log_{10} to 1.6 log_{10} (99.98 to 97\%) for \textit{E. coli}. Geometric mean reductions of these microbes in the non-vegetated SSF control reactor were significantly lower than in the vegetated SSF reactor, indicating that the presence of vegetation in the SSF wetland had a positive effect on bacterial reductions. These data support the conclusions of previous research on the effects of wetlands vegetation on enteric microbe reductions in SSF treatment wetlands (Gersberg et al., 1989; Soto et al., 1999; Warren et al., 2000).

HRT in the laboratory-scale reactors varied from 2 to 4 days in the SF reactor, 4 to 17 days in the SSF wetland reactor, and 4 to 17 days in the SSF control reactor. Corresponding volumetric decay rates ($k_{v,30}$) for fecal coliforms varied from 0.4 to 0.9 d^{-1} in the SF wetland, 0.6 to 1.4 d^{-1} in the SSF wetland and 0.5 to 1.1 d^{-1} in the SSF control reactor. Areal decay rates ($k_{a,30}$) for fecal coliforms were 2.9 to 6.3 cm d^{-1} in the SF wetland, 9.1 to 20 cm d^{-1} in the SSF wetland and 8.1 to 15 cm d^{-1} in the SSF control. Reduction rates for \textit{E. coli} and fecal coliforms were similar. \textit{S. typhimurium} volumetric decay rates were lower than those for \textit{E. coli} and fecal coliforms, varying from 0.2 to 0.5 d^{-1} in the SF wetland, 0.5 to 0.8 d^{-1} in the SSF wetland, and 0.3 to 0.6 d^{-1} in the SSF control. Areal decay rates for \textit{S. typhimurium} were 1.4 to 3.6 cm d^{-1} in the SF wetland, 6.8 to 11 cm d^{-1} in the SSF wetland and 5.6 to 8.0 cm d^{-1} in the SSF control. The areal decay rates calculated for \textit{Salmonella}, fecal coliforms and \textit{E. coli} were higher for the field SF wetland than in the laboratory-scale SF wetland reactor, possibly due to the effects of environmental conditions not present during the laboratory study (e.g., sunlight irradiance) or differences in wetland design (e.g., two-cell vs. single-cell, multiple vegetation species vs. monoculture).
Conclusions

Significant reductions of enteric microbes can be achieved in SF treatment wetlands. The field-scale and laboratory-scale surface flow systems of the present study were operated as secondary treatment systems (i.e., receiving wastewater from primary treatment anaerobic lagoons). As such, the results of this study show that a secondary treatment system using surface flow constructed wetlands can be effective for achieving significantly greater pathogen reductions in swine wastewater than would be achieved using a single-stage lagoon system. Of the microbial indicators studied, fecal coliforms and *E. coli* appeared to be the best indicators for removal of *Salmonella* in the SF wetland system. The viral indicators and *C. perfringens* spores were removed to a similar extent as the enteric bacteria studied, indicating that SF constructed wetlands may be as effective for reducing concentrations of viral and parasitic pathogens as for bacterial pathogens.

The results from the laboratory study show that loading rate is an important variable to consider when designing surface or subsurface flow wetland treatment systems to remove pathogens from wastewater. The data also indicated that SSF systems, whether vegetated or not, can achieve greater pathogen reductions than similarly sized SF wetlands operated at the same loading rates. The presence of vegetation in the SSF wetland significantly improved the removal of *Salmonella*, fecal coliforms and *E. coli* compared to the non-vegetated SSF control reactor, thus supporting previous research reporting positive effects of vegetation on enteric microbe removal in treatment wetland systems.

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References


