The effect of wetland vegetation on the survival of *Escherichia coli, Salmonella typhimurium*, bacteriophage MS-2 and polio virus

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ABSTRACT

A study was conducted to examine the role of aquatic plants used in constructed wetlands on the survival of enteric bacteria and viruses. Four small-scale wetland systems, receiving fresh water and two other wetland systems, receiving secondary unchlorinated sewage were used in this study. Fresh water and secondary sewage without the presence of any aquatic plants were used as controls. *Escherichia coli*, *Salmonella typhimurium,* bacteriophage MS-2 and poliovirus were added to the waters collected from the wetlands and controls. The presence of aquatic plants significantly increased the die-off of both bacteria in fresh water and secondary sewage. No significant difference in the die-off of *E. coli* and *S. typhimurium* was observed in water from wetlands with different types of plants in freshwater. However, there was a significant difference in the die-off of *E. coli* in water with aquatic plants when sewage was used. The presence of the plants significantly increased the inactivation of MS-2 and poliovirus. Additional work on the survival of *E. coli* indicated that the plausible mechanism of bacterial die-off in constructed wetlands is through increased microbial competition or predation.

Key words | bacteriophage MS-2, constructed wetland, *E.coli*, poliovirus, *S. typhimurium*, survival, wastewater

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INTRODUCTION

Recently, attention has been focused on the ability of wetlands to efficiently reduce human pathogens from wastewater. There have been several studies published on microbial water quality improvement using wetlands (John 1984; Gersberg *et al.* 1987*a*; Gearheart *et al.* 1989; Falabi 1996; Karpiscak *et al.* 1996; Mandi *et al.* 1996; Green *et al.* 1997). John (1984) reported 99 percent removal of coliforms, *E. coli* and streptococci, from a water hyacinth covered lagoon in Malaysia. Karpiscak *et al.* (1996) reported 57 percent reduction of total coliforms, 62 percent reduction of fecal coliforms, and 38 percent reduction of coliphage from a duckweed pond. In a multi-species (bulrush, cattail, black willow, and cottonwood) wetland system, Karpiscak *et al.* (1996) reported reduction of total and fecal coliforms by 98 and 93 percent, respectively.

The efficiency of removal/inactivation of total coliforms in gravel-based constructed wetlands was examined by Gersberg *et al.* (1989*a*). The wetlands were planted with bulrush and received primary municipal wastewater. The removal of total coliforms by the vegetated wetland was 99.1 percent. In a study at Santee, California, the mean removal efficiency of bacteriophage MS-2 was nearly 99 percent. Removal by the vegetated wetland was greater than the unvegetated wetland (Gersberg *et al.* 1987*a*). In another study, MS-2 was continuously seeded into an experimental marsh cell at Arcata, California dominated by *T. latifolia* and *S. lacustris* (Gersberg *et al.* 1989*b*). Bacteriophage MS-2 removal efficiency ranged from 79 percent in April to 96 percent in August and September with a mean removal efficiency of 91.5 percent.

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Removal of total coliform, fecal coliform and *Enterobacteriaceae* from five constructed wetlands was studied in the Czech Republic (Ottoval *et al.* 1997). The reduction of total coliforms was about 99 percent with the exception of one of the wetlands. The reduction of fecal coliforms ranged from 95.3 percent to 99.9 percent, and reduction of *Enterobacteriaceae* ranged from 94.7 percent to 99.5 percent. Ottoval *et al.* (1997) concluded that the lower reduction (85.9%) of total coliforms observed in one the wetlands could be due to the difference in vegetation and the difference in retention times. A two-stage system for treating high-strength wastewater from an abattoir at Pachuca, Mexico was studied by Rivera *et al.* (1997). The system consisted of an anaerobic digester followed by a constructed wetland. The wetland had a horizontal subsurface flow through the root zone of *Phragmites australis* and *Typha latifolia* planted in a gravel structure. The mean removal efficiency for both total and fecal coliforms from this system for a one-year period was greater than 99 percent.

None of the reported studies examined the mechanism of pathogen reduction in vegetated wetlands. The objective of this study was to assess the effect of different aquatic plants on the die-off of *Escherichia coli*, *Salmonella typhimurium*, bacteriophage MS-2 and poliovirus type 1 in wetlands in a controlled environment.

MATERIALS AND METHODS

Organisms

Escherichia coli ATCC (American Type Culture Collection) 15597 and *Salmonella typhimurium* ATCC 23564 were used in this study. Both *E. coli* and *S. typhimurium* inocula were prepared by centrifuging (1,000 × g for 10 min) late-log phase cultures grown in tryptic soy broth (TSB; Difco, Detroit, MI) at 37°C. Bacterial cells were washed twice with sterile Tris buffered saline (Trizma base, Sigma Chemical, St. Louis, MO) solution. After the final wash, cells were suspended in the sterile buffer and diluted to a concentration of 2.5×10^6 to 5×10^6 CFU (Colony Forming Unit) per ml. Mcfarland standard 0.5 was used as a reference of tubidity to achieve a bacterial concentration of 2.5×10^6 to 5×10^6 CFU per ml.

Preparation of stock MS-2 and poliovirus

Escherichia coli (ATCC 15597) was grown overnight in tryptic soy broth (TSB; Difco, Detroit, MI) at 37°C without shaking. This culture was used to inoculate fresh TSB. The inocula were incubated for 3–4 h at 37°C with continuous shaking to obtain an exponential growth of the bacteria. MS-2 (ATCC 15597B) was serially diluted in Tris buffer pH 7.4 (Trizma base; Sigma, St Louis, MO) to a concentration of 10^5 PFU (Plaque Forming Unit) per ml. One ml of an exponential culture of *E. coli* and 0.1 ml MS-2 were added to tubes of molten overlay agar (TSB with 1% agar) and mixed. The mixture was poured into tryptic soy agar plates (TSA; Difco). After incubating 24h at 37°C, 6 ml Tris buffer was added to the plates and incubated for 2 hours at room temperature for separation of the phage particles. The liquid portion was aspirated off from the plates and centrifuged. The pellet was discarded and the supernatant was filtered using 0.22 μm filter and stored at 4°C. The MS-2 stock was titrated prior to use in the survival experiments. Poliovirus type 1 (strain LSc-2ab) was propagated on the Buffalo Green Monkey (BGM) Kidney cells.

Survival studies: fresh water wetland systems

Four small-scale artificial wetlands were constructed at the Environmental Research Laboratory of The University of Arizona. The first wetland system (wetland 1) was composed of three metallic tanks connected to each other. Each container held approximately 200 gallons of water. *Typha domengensis* and *Iris laevigata* were grown in the first container. Rocks were used to provide mechanical support to the plants. *Typha domengensis* and *Calocasia esculenta* were grown in soil in the second container. The third container did not contain any plants. Non-chlorinated potable water (fresh water) was used as a water source and the water was continuously circulated between these three containers using a submerged pump. The plants were planted in January and grown for six months in this system. The second wetland system (wetland 2) was constructed using a plastic container with a capacity of 700 litres of water. Potable water was used as a water source and *Lemna minor* was grown in this container for six months. The third wetland system (wetland 3) was similar to the second wetland system,

other than it contained three different plants, *Typha domengensis, Lemna minor,* and *Elodea densa*. The fourth wetland system (wetland 4) was built using a 200 litre plastic container and contained only *Elodea densa*, a submerged plant. The first wetland system was built in an open environment, and the second, third and the fourth wetlands were built inside a greenhouse. Plants were grown for six months in these wetlands. Forty millilitres of water was collected from each of these wetlands in sterile polypropylene centrifuge tubes, stored in an ice cooler and transported to the laboratory. The water samples were then inoculated with either *E. coli* or *S. typhimurium* at a concentration of 10^6 CFU/ml. Fresh water without the presence of any aquatic plants was used as a control. *E. coli* survival experiments were conducted in triplicate and *S. typhimurium* in duplicate at room temperature (23°C). After various periods of time a 0.3 ml sample was withdrawn from the tubes and the concentration of bacteria were enumerated. Survival experiments for bacteriophage MS-2 and poliovirus were done in a similar manner with water from two of the wetlands, (wetland 1 and wetland 2). The experiments were conducted in duplicate at room temperature.

Survival studies: wetlands receiving sewage

Water samples were collected from the Constructed Ecosystem Research Facility (CERF) located in Pima County, Tucson, Arizona. The operation of CERF began in 1989 with the intention of studying the ability of constructed wetlands to be used for treatment of wastewater (Karpiscak 1996). CERF receives secondary unchlorinated wastewater from the adjacent Roger Road Municipal Wastewater Treatment Facility. The facility has six raceways lined with 30 mil hyperlon. Water samples were collected from two of the six ponds (wetlands). The first pond (wetland 5) contained water hyacinth (*Eichhornia crassipes*) and the pond was 20 m in length, 8.2 m in width, and 0.6 m in depth, receiving wastewater at a rate of 58 L per minute. The second pond (wetland 6) contained duckweed and the pond was 65 m in length, 11.9 m in width, and 0.9 m in depth with a flow rate of 55 L per minute. Forty ml of sewage was collected from these wetlands in sterile polypropylene tubes, stored in an ice cooler and transported to the laboratory. The water samples were inoculated with either *E. coli*, *S. typhimurium,* MS-2 or poliovirus at similar concentrations described for the freshwater wetland studies and was kept at room temperature (23°C). Wastewater from the inlet of the ponds was used as a control. After various periods of time a 0.3 ml sample was withdrawn from the tubes and the concentration of bacteria were enumerated.

Survival studies: sterile water

E. coli or MS-2 was added to a concentration of 10^6 CFU or PFU/ml to 40 ml of nonsterile, autoclaved or filter sterilized (pore size, 0.2 μm) water collected from the first freshwater wetland system (wetland 1) containing aquatic plants *Typha domengensis, Iris laevigata,* and *Calocasia esculenta*. The experiment was conducted at room temperature.

Enumeration methods

E. coli was assayed by spread plate method using mFC agar (Difco Laboratories, Detroit, MI). The plates were incubated at 44.5°C for 24 h. *S. typhimurium* was enumerated using Hektoen enteric agar (Difco). Heterotrophic bacteria were enumerated using R2A agar (Difco, MD). MS-2 was assayed by the double layer method described by Adams (1959). The host strain used for the assay was *Escherichia coli*, strain ATCC 15597. Poliovirus was enumerated by plaque assay method on Buffalo Green Monkey kidney (BGM) cells. Determination of pH, turbidity and total dissolve solids was performed according to the procedures described in the *Standard Methods for the Examination of Water and Wastewater* (APHA 1995).

Data analysis

Linear regression analyses were used to calculate die-off rates ($\log_{10}$ reduction per day) by the following equation: $\log_{10} \mathrm{Nt/No} = -\mathrm{mx} + \mathrm{b}$, where $\log_{10}$ Nt/No is the ratio of the $\log_{10}$ value at time t (measured in days) to the initial $\log_{10}$ value (log No), x is the time in days, b is the intercept value, and m is the slope. Analyses of variance and regression analyses were performed using the SAS statistical software (SAS Institute Inc., Cary, NC, version 12). Spearman correlation was performed for non-normally distributed data (Cody & Smith 1991).

RESULTS

Survival studies: fresh water wetland systems

The survival of *E. coli* and *S. typhimurium* in water from wetlands and control (without any vegetation) receiving fresh water are shown in Figures 1 and 2, respectively. The presence of aquatic plants significantly increased the die-off of *E. coli* ($p < 0.01$) and S. *typhimurium* ($p < 0.01$) compared to the control. *E. coli* decreased 3.4 orders of magnitude in 6 days in fresh water without the presence of any aquatic plants. In the wetland containing plants, *E. coli* decreased 5 $\log_{10}$ in wetland 1, 5 $\log_{10}$ in wetland 2, 5.3 $\log_{10}$ in wetland 3 and 4.7 $\log_{10}$ in wetland 4 in six days. *S. typhimurium* decreased 3.2 $\log_{10}$ in 6 days in the control compared with 4.8 $\log_{10}$, 4.5 $\log_{10}$, 4.3 $\log_{10}$, and 4.19 $\log_{10}$ reductions in wetlands 1, 2, 3, and 4, respectively. The results suggest that the presence of aquatic plants in fresh water wetlands enhanced the die-off of both bacteria. However, no significant difference in the die-off of *E. coli* and *S. typhimurium* was observed between the wetlands containing different plants. The results in Table 1 show the physical and chemical characteristics and heterotrophic bacterial count of the water samples. None of the physicochemical parameters were significantly related to the die-off of *E. coli* in the survival studies. The die-off rates ($\log_{10}$ day^{-1}) of *E. coli* and *S. typhimurium* are presented in Table 2. The data for the die-off rates of both bacteria fit the usual die-off model ($\log_{10}$ Nt/No $= -$mx $+$ b, where $\log_{10}$ Nt/No is the ratio of the $\log_{10}$ value at time t, to the initial $\log_{10}$ value, x is the time in days, b is the intercept value, and m is the slope). The die-off of both bacteria in wetlands containing aquatic plants was higher compared to the control. The highest die-off of 1.07 $\log_{10}$ day^{-1} for *E. coli* and 1.16 $\log_{10}$ day^{-1} for *S. typhimurium* was observed in the multiple species wetland (wetland 1).

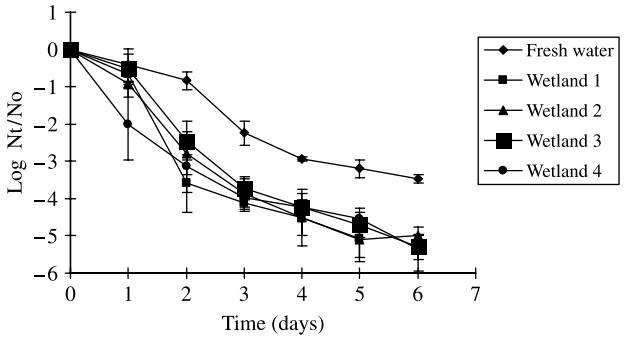

Figure 1 | Survival of *E. coli* in fresh water wetlands.

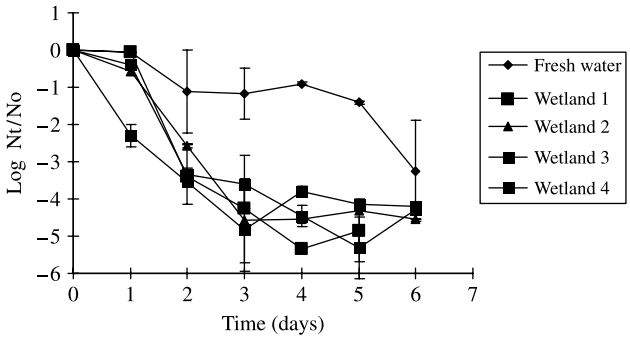

Figure 2 | Survival of *S. typhimurium* in fresh water wetlands.

Survival of MS-2 and poliovirus in wetlands receiving fresh water is illustrated in Figures 3 and 4, respectively. The presence of aquatic plants significantly increased the inactivation of MS-2 ($p = 0.05$) and poliovirus ($p = 0.002$). No significant difference in virus inactivation was observed between wetlands containing different plants. Similar results were obtained for poliovirus. The inactivation rates are shown in Table 3. The inactivation of both MS-2 and poliovirus were higher in water from the vegetated wetlands compared to the control.

Table 1 | Physical and chemical characteristics of wetland water

Wetlands	pH	Turbidity (NTU)	Total dissolved solid (mg/L)	Heterotrophic bacteria CFU/ml
Fresh water control	7.3	0.2	590	1.3E + 02
Wetland 1	7.9	0.4	316	2.2E + 05
Wetland 2	7.7	0.8	447	2.1E + 05
Wetland 3	7.4	0.6	403	5.5E + 04
Wetland 4	6.9	1.4	189	NS
Sewage control	7.4	1.9	307	7.4E + 06
Wetland 5	7.0	1.8	503	2.0E + 06
Wetland 6	7.4	2.9	493	7.7E + 06

NS = no sample.

Table 2 | Die-off rates (k) of *E. coli* and *S. typhimurium* in wetlands receiving fresh water and sewage

Wetland	*E. coli* die-off rates (log_{10} day^{-1})	*R*-square values	*S. typhimurium* die-off rates (log_{10} day^{-1})	*R*-square values
Fresh water control	0.644*	0.952	0.435*	0.758
Wetland 1	1.070*	0.882	1.169*	0.845
Wetland 2	0.897*	0.912	0.882*	0.789
Wetland 3	0.931*	0.916	0.851*	0.801
Wetland 4	0.726*	0.847	0.589†	0.605
Sewage control	0.541*	0.811	0.648*	0.717
Wetland 5	0.827*	0.910	0.902*	0.908
Wetland 6	0.820*	0.848	0.613†	0.598

*$p = <0.01$.
†$p = <0.05$.

Survival studies: wetlands receiving sewage

There was a significant difference ($p < 0.01$) in the die-off of *E. coli* but *not S. typhimurium* in wetlands receiving sewage effluent. *E. coli* decreased 5 log_{10} and 5.2 log_{10} in 6 days in wetlands containing water hyacinths (wetland 5) and duckweed (wetland 6), respectively, compared with a 3.9 log_{10} reduction in sewage. *S. typhimurium* decreased 6.1 log_{10} and 3.8 log_{10} in 6 days in wetlands containing water hyacinth and duckweed, respectively, compared with a 4.6 log_{10} reduction in the control. The die-off rates of *E. coli* were higher (Table 2) in wetlands containing aquatic plants compared with the control. However, the die-off rates of *S. typhimurium* were higher (Table 2) in wetland 5 and slightly lower in wetland 6 compared with the control.

There was a significant difference ($p = 0.02$) in the inactivation of MS-2 in the wetlands receiving secondary sewage. A greater rate of inactivation was observed in the wetland containing duckweed (*Lemna minor*) compared with the wetland containing water hyacinth (*Eichhornia crassipes*). However, poliovirus inactivation in these wetlands was not significantly different (Table 3).

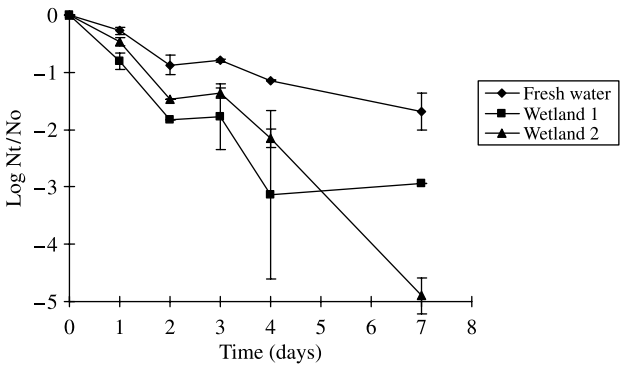

Figure 3 | Persistence of bacteriophage MS-2 in fresh water wetlands.

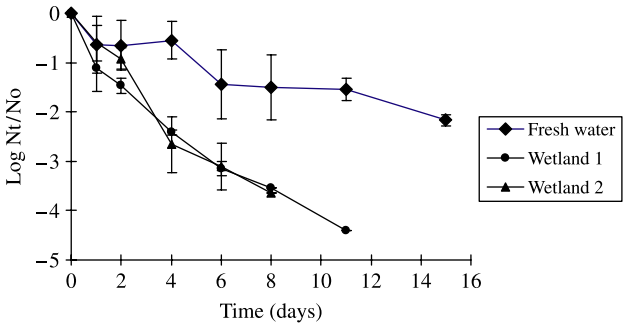

Figure 4 | Persistence of poliovirus in fresh water wetlands.

Table 3 | Inactivation rates (k) of MS-2 and poliovirus in wetlands receiving fresh water and sewage

Wetland	MS-2 inactivation rates (log_{10} day^{-1})	*R*-square values	Poliovirus die-off rates (log_{10} day^{-1})	*R*-square values
Fresh water control	0.214*	0.919	0.144*	0.765
Wetland 1	0.341*	0.683	0.361*	0.978
Wetland 2	0.714*	0.953	0.454*	0.931
Sewage control	0.310*	0.989	0.363*	0.938
Wetland 5	0.232	0.755	0.375*	0.999
Wetland 6	0.309*	0.967	0.282*	0.948

*$p = <0.01$.

Survival of *E. coli* and MS-2 in non-sterile, filtered and autoclaved wetland water

The survival of *E. coli* and MS-2 in non-sterile, filter sterilized, and autoclaved wetland water is shown in Figures 5 and 6, respectively. The survival of *E. coli* in both filter sterilized and autoclaved wetland water was similar and remained largely unchanged throughout the experiment. However, *E. coli* die-off was significantly greater in non-sterile wetland water, resulting in the decrease of 4.5 $\log_{10}$ in 5 days. The die-off of *E. coli* in non-sterile wetland water was similar to that observed in our previous experiments. These results indicate that bacterial die-off in the wetland could be due to microbial competition or predation. Persistence of MS-2 in non-sterile, filter sterilized and autoclaved water was different from that observed for *E. coli*. MS-2 was inactivated rapidly in both non-sterile and filtered water compared to the autoclaved water. These results suggest that bacteriophage inactivation could be enhanced in wetlands due to the presence of microbial metabolites. It is plausible that the autoclave sterilization altered the chemical nature of the metabolites, resulting in a decrease in inactivation.

DISCUSSION

Under natural conditions where aquatic plants are present, a reduction of total and fecal coliform bacteria has been reported by a number of previous studies (Gersberg *et al.* 1989*b*; Karpiscak *et al.* 1996; Thurston *et al.* 2001). The results of the present study support these findings. In the present study when aquatic plants were present higher

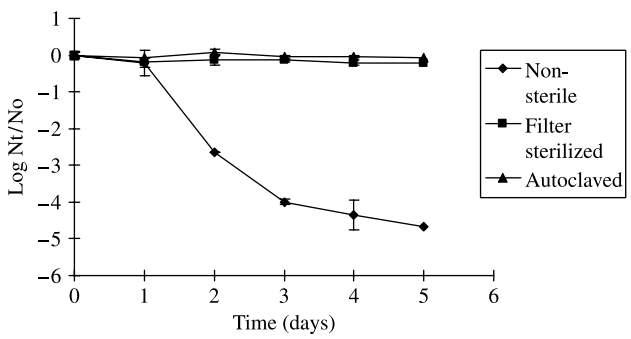

Figure 5 | Survival of *E. coli* in non-sterile, filter sterilized and autoclaved wetland water.

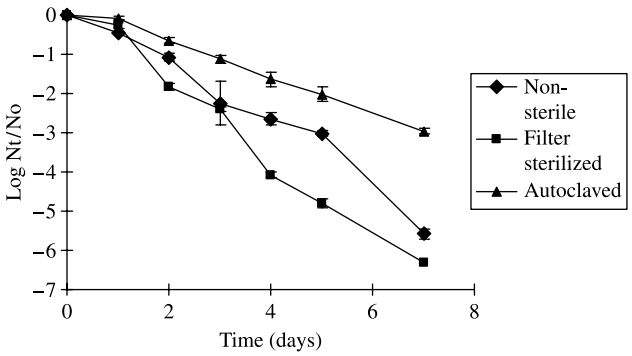

Figure 6 | Persistence of bacteriophage MS-2 in non-sterile, filter sterilized and autoclaved wetland water.

die-off rates for *E. coli* and *S. typhimurium* were observed in water from wetlands receiving fresh water. This may have been due to the increased competition for limited nutrients or trace elements with natural microorganisms, attack by lytic phage and bacteria or predation by nematodes, protozoa or ciliates. Predation is thought to play an important role in the removal of bacteria from wastewater in constructed wetlands (Mandi *et al.* 1993; Green *et al.* 1997; Decamp & Warrren 1998). Mandi *et al.* (1993) suggested predation by nematodes as an important factor in the removal of fecal coliforms from wastewater in macrophyte ponds. Bacteriovorous activity of protozoa (Green *et al.* 1997) and cilliates like *Paracmecium spp, Oxytrichids, Halteriain, Plagiopyla* and *Caenomorpha* in wetlands was reported by Decamp & Warren (1998). Bacterial growth can also be reduced by inhibitors produced by algae (Chrost 1972, 1975).

Plants excrete photosynthate and other organic compounds through their roots. The high concentration of bacteria in the rhizosphere zone is well documented. It is thought that photosynthate and other compounds released by plants provide nutrients for the rhizosphere microorganisms resulting in their proliferation. The presence of aquatic plants in wetlands might create a nutrient rich environment in the rhizosphere zone, causing an increase in natural flora. The absence of microorganisms in both filter sterilized and autoclaved wetland water did not appear to affect the survival of *E. coli*. However, E. *coli* die-off was significantly greater in non-sterile wetland water, resulting in a decrease of 4.5 $\log_{10}$ in 5 days. These results indicate that a plausible mechanism of microbial die-off observed in

the vegetated wetland systems could be due to biological antagonism or predation. A similar observation reported by Riser *et al.* (1985) indicated that seeded S. *typhimurium* grew rapidly in a sterile nutrient solution, whereas growth appeared to be suppressed in a non-sterile nutrient solution. The authors concluded *S. typhimurium* could not compete favorably with the normal flora. The heterotrophic bacterial count in freshwater wetlands was 2–3 logs higher than the control, but there was no marked difference in bacterial numbers between the wetlands receiving sewage. However, no significant correlation was found between the bacterial die-off and heterotrophic bacteria numbers.

The reduction of bacteriophage and enteric viruses in vegetated wetlands has been previously reported (Gersberg *et al.* 1987*a*,*b*; Karpiscak *et al.* 1996). Our experiments confirmed these results. MS-2 and poliovirus were inactivated at a greater rate in wetlands receiving freshwater. These results suggest that bacteriophage inactivation could be enhanced in wetlands due to the presence of microbial metabolites or the presence of proteolytic substances released by microbes or plants. Certain bacteria release proteolytic enzymes, which are capable of destroying the protein capsid of the viruses, or may release other substances, which enhance virus inactivation (Cliver & Hermann 1972). The presence of aquatic plants in vegetated wetlands may enhance rhizosphere bacterial populations, which might cause the inactivation of viruses. Bacteria have been found to affect the persistence of MS-2 in groundwater (Yates *et al.* 1990).

More than 150 plant species have been reported to contain virus inhibitory substances (Meyer *et al.* 1995). The antiviral protein of *Phytolacca americana* (PAP) was found to inhibit the infectivity of plant viruses (Chen *et al.* 1991). A plausible mechanism of virus inactivation in vegetative wetlands could be through the antiviral properties of aquatic plants.

In wetlands containing sewage, a greater die-off of *E. coli* was observed when aquatic plants were present. However the die-off of *S. typhimurium* was not significantly different between the wetlands. The die-off rates of *E. coli* and *S. typhimurium* were similar in the freshwater wetlands. However, the die-off rate of *E. coli* was greater in wetland 6 compared to *S. typhimurium* and the opposite was observed in wetland 5.

There was no significant difference in virus inactivation in freshwater wetlands containing different aquatic plants. However, a significant difference in MS-2 inactivation was observed in wetlands receiving secondary sewage. The wetland containing *Limna minor* had a greater inactivation compared to the wetland containing *Eichhornia crassipes*. However, poliovirus inactivation in wetlands receiving sewage was not influenced by the presence of plants. The inactivation rates of both MS-2 and poliovirus in the fresh water wetland were greater than the wetlands receiving sewage. This may have been due to the fact that the inactivation of virus in vegetated wetlands might be specific to virus and plant species. Moreover, the turbidity of the sewage was much higher than the water from freshwater wetlands. Particulate matter may exert a protective effect on the viruses in the wetlands. Presence of particulate matter was found to decrease the inactivation of virus in seawater (Gerba & Schaiberger 1975). A plausible cause why poliovirus inactivation in sewage was not influenced by the presence of plants could be due to the presence of particulate matter and other organic matter in the sewage, which may exert a protective effect on the virus.

Comparison of results with field studies

Several field studies reported the reduction of indicator bacteria, bacteriophage and virus in vegetated wetlands (John 1984; Gersberg *et al.* 1987*b*; Gersberg *et al.* 1989*b*; Karpiscak *et al.* 1996; Mandi *et al.* 1996; Green *et al.* 1997). All of these studies suggested the reduction of microorganisms in vegetated wetlands, but only a few performed a comparative study on the removal/reduction of microorganisms in the presence and absence of vegetation (Gersberg *et al.* 1987*a*; Quinonez-Diaz *et al.* 2001). Removal of total coliforms and coliphage by vegetated wetlands was higher than the unvegetated wetland (Gersberg *et al.* 1987*a*, 1989*b*). Our results suggest a greater reduction of bacteria and bacteriophage in the presence of aquatic plants. Greater die-off of *E. coli* and *S. typhimurium* were observed in water of vegetated wetlands. In contrast to our study, the survival of fecal coliform in a wastewater retention reservoir containing *Lemna gibba* remained constant for 5 days (Dewedar 1995). The experiment was conducted using dialysis sacs filled with a suspension of fecal coliform bacteria. Thus, the inoculated

bacteria were not in contact with the natural flora as well as not being exposed to natural predators. The present study indicated that the use of membrane diffusion chambers to determine bacterial die-off in wetlands might not reflect actual conditions. The use of membrane diffusion chambers to determine bacterial die-off has also been previously criticized (Springthorpe *et al.* 1993).

The removal of indicator bacteria, coliphage and enteric viruses in an unvegetated wetland cell was greater than the removal of the vegetated cell (Quinonez-Diaz *et al.* 2001). The author concluded that exposure to sunlight in the unvegetated cell resulted in higher removal than from the vegetated cell. However, the difference observed was not statistically significant. In contrast to that study, our experiment was performed in a controlled environment, which suggested a statistically significant difference between the vegetated and unvegetated wetlands, indicating a greater die-off from the vegetated wetlands. Karpiscak (1996) reported a 98 percent and 93 percent reduction of total and fecal coliforms, respectively, from a multispecies wetland. The average retention time in that wetland was 3.8 days, indicating less than one $\log_{10}$ reduction of indicator bacteria per day. Quinonez-Diaz *et al.* (2001) reported an overall 90 percent (equivalent to one $\log_{10}$) reduction of indicator bacteria and coliphage in one to two days. Rivera *et al.* (1997) observed a greater than 99 percent (equivalent to 2 $\log_{10}$ reduction) removal of fecal coliforms with a retention time of 1.7 days from a wetland planted with *Phragmities australis*. The die-off rates of *E.coli* and *S. typhimurium* observed in our study ranged from 0.72 $\log_{10}$ to 1.07 $\log_{10}$ day^{-1} and 0.58 $\log_{10}$ to 1.16 $\log_{10}$ day^{-1}, respectively. The inactivation rates of MS-2 and poliovirus in our study was less than one $\log_{10} day^{-1}$. The survival of bacteriophage and poliovirus in artificial wetlands was studied by Gersberg *et al.* (1987). Bacteriophage removal in vegetated wetlands was significantly greater than the unvegetated wetland. The results suggested the superiority of treatment by the vegetated wetland compared to the unvegetated wetland. The decay of the seeded MS-2 followed a first order kinetics with a decay rate of 0.025 to 0.028 PFU per hour during the summer. The decay rate was lower (0.012 PFU per hr.) during the winter month. The decay rates observed in our study were roughly similar to the decay reported by Gersberg *et al.* (1987).

CONCLUSION

The present study suggests that biological antagonism such as predation, microbial competition, attack by lytic bacteria and phages, or modification of micro-environment by microbial metabolism may play a significant role in the reduction of bacteria and virus in wetlands. However, other processes, such as sunlight, sedimentation, absorption and natural die-off are also likely to play an important role in bacterial die-off. Future studies are needed to determine the exact mechanism of microbial antagonism in wetlands.

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