Microbiological assessment of ambient waters and proposed water sources for restoration of a Florida wetland

W. Q. Betancourt and J. B. Rose

ABSTRACT

This study evaluated the microbial quality of reclaimed and storm water as proposed sources for restoration of a Florida wetland. Bacterial indicators, bacteriophages and waterborne pathogenic microorganisms (*Cryptosporidium*, *Giardia* and infectious enteric viruses) were analysed during a 1-year period in order to determine potential public health risks associated with exposure to the proposed water sources for restoration. Ambient waters within the wetland (four active water wells and four major lakes) were included in the study in order to determine the microbial water quality before restoration. Storm water and lakes had the highest level of microbial contamination. Much lower levels of microbial indicators and waterborne pathogens were found in reclaimed water and groundwater. Pathogen occurrence in groundwater was intermittent. Owing to the small percentage of source waters (3.3%) migrating to the water wells, ambient concentration of microbial constituents in surface and groundwater could dominate microbial risk. The results of this study indicate that, in the light of the uncertainties involved in computing average *Cryptosporidium* concentrations, additional characterization of the current ambient water quality should be ongoing prior to restoration.

Key words | *Cryptosporidium*, *Giardia*, enteric viruses, microbial indicators, wetland, restoration

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INTRODUCTION

The United States Environmental Protection Agency, the Army Corps of Engineers and the National Resource Conservation Service define wetlands as areas that are inundated or saturated by surface or groundwater at a frequency and duration sufficient to support and that under normal circumstances do support, a prevalence of vegetation typically adapted for life in saturated soil conditions.

The many benefits that wetlands provide have been extensively recognized and programmes have been developed to restore and protect wetland resources at the local, state and federal levels of government (www.epa.gov/OWO/wetlands/science/hgm.html).

As the link between land and water, wetlands play a vital role in water quality management programmes. Wetlands provide a wide array of functions including conveyance and storage of floodwater, nonpoint source runoff filtration, and prevention of erosion and saltwater intrusion, which directly benefit adjacent and downstream waters. In addition, wetlands provide important biological habitat, including nursery areas for aquatic life and wildlife, and other benefits such as groundwater recharge and recreation.

Wetlands are present in landscapes that favour the pounding or slow runoff of surface water, discharge of groundwater, or both. Some wetlands depend almost exclusively on precipitation, surface water or groundwater, but most wetlands receive water from a combination of these sources. Studies on selected wetlands and their contiguous watersheds in four landscapes in the US indicated that their sources of water are appreciably different and that management or protection of these wetlands would require different managements (Winter *et al.* 2001).

Wetlands are estimated to make up about 6% of Earth; Florida contains approximately 20% of the remaining wetlands in the US (http://sjr.state.fl.us). Draining, filling, embankments, reservoirs, diversions or groundwater pumping has obliterated nearly 50% of the nation's original wetlands. This has become another component of the global environmental crisis along with the destruction of forests or the ozone layer (Denison 1997).

A Florida wetland whose ecological functions and aesthetic conditions have been affected by development, groundwater pumpage and drought conditions was chosen as a study site to evaluate the feasibility of restoring its hydrologic conditions using reclaimed water and/or storm water. Microbial indicators of water quality and waterborne pathogens were analysed during a 1-year study in order to determine potential public health risks associated with microbial contaminants in proposed water sources for restoration. The main objectives of this study were: (i) to assess the microbial quality of the potential water sources (storm water and reclaimed water) proposed for wetland and lake restoration; (ii) to assess the microbial quality of ambient waters (lakes and water wells) within the wetland; and (iii) to identify microbiological constituents of concern for the risk assessment plan of study.

Work elements in support of the public health risk assessment included site characterization, computer-based hydrologic modelling and ambient water quality sampling. This work focuses on the microbiological characterization of the proposed water sources for restoration and ambient waters of the wetland (lakes and groundwater).

METHODS

Water samples

Storm water and reclaimed effluent samples were collected respectively from canals associated with a major drainage area located within the wetland and from a storage tank at a water reclamation facility that provides secondary and advanced treatment to wastewater. In order to determine the microbial status of ambient waters within the wetland, water samples from four of the major lakes and four active water wells were collected. Physical chemical parameters such as pH, turbidity and temperature were determined during sample collection.

Assessment of microbial indicators and waterborne pathogens

Grab samples for microbial indicators were collected using sterile 1,000-ml plastic bottles, placed on ice and transported to the laboratory for analysis within 4 hours of collection. Bacterial indicators analysed included total and fecal coliforms, enterococci and *Clostridium perfringens*. Water samples were filtered through membrane filters (0.45 μm [pore size] and 47 mm diameter; Gelman Sciences) according to procedures described in the *Standard Methods for the Examination of Water and Wastewater* (1998). Following filtration, membrane filters were placed on specific media and incubated at specific temperatures depending on the type of bacterial indicator evaluated. The concentration of bacteria was expressed as colony forming unit (CFU) per 100 ml (*Standard Methods for the Examination of Water and Wastewater* 1998).

Total coliform and fecal coliform bacteria were detected on M-Endo medium and mFC medium (Difco Laboratories, Detroit, Michigan), respectively. Enterococci were enumerated on mEI agar (Difco Laboratories, Detroit, Michigan).

Clostridium perfringens was enumerated by growth on mCP medium (Acumedia, Baltimore, Maryland). During the study, sample sizes were increased to 20 and 50 ml since lower densities of *C. perfringens* are found in environmental waters. Membrane filters were incubated anaerobically in GasPak jars (BBL [Baltimore Biological Laboratories] GasPak, Beckton Dickinson) for 18 to 24 hours at 45 ± 0.5°C. Yellow colonies were exposed to ammonium hydroxide fumes; colonies that turned red or dark pink were enumerated as *C. perfringens* (Bisson & Cabelli 1979).

Bacteriophages

Bacteriophages were analysed by two methods: the agar overlay method of Adams (1959) and the enrichment protocol obtained from Bill Yanko (Director of Wastewater, County Sanitation Districts of Los Angeles County, San Jose Creek Water Quality Laboratory, Whittier, California). Two bacterial hosts were used separately to analyse the samples:

Escherichia coli host bacterial culture (host strain ATCC [American Type Culture Collection] 15597) and *E. coli* HS (pFamp)R (male specific coliphage host).

The host bacterium, *E. coli* (ATCC strain 15597) was used in the agar overlay method. Cultures were grown to logarithmic phase in tryptic soy broth (TSB) at 37°C for 4 to 6 hours under constant agitation. Aliquots of 2 ml were mixed with molten trypticase soy agar (TSA) (3 ml) and 1 ml of host bacteria. The mixture was then poured on solid plates of TSA, allowed to solidify and incubated for 24 h at 37°C. Plates were observed for plaque formation and scored. Five or ten replicates of each sample were analysed to reduce the limit of detection to 10 and 5 plaque-forming units (PFU per 100 ml), respectively.

The enrichment protocol (presence/absence test) was used to assess the potential for viruses to affect the water wells. This assay increases the number of phages to the point that they can be detected by direct plating; therefore it was also applied to analyse those surface water samples that were negative with the agar overlay method. The host bacterium *E. coli* HS p(Famp)R was used for enumerating F-specific or male-specific bacteriophages. F-specific bacteriophages can be further characterized as F + RNA or F + DNA bacteriophages and used as indicators of groundwater vulnerability to contamination (USEPA 1999). HS Famp + was also employed in the agar overlay method.

E. coli p(Famp)R cultures were grown, maintained and assayed on tryptone medium containing ampicillin and streptomycin as described by Debartolomeis & Cabelli (1991). Media preparation followed modifications described by Yanko *et al.* (1999). Briefly, 11X broth medium used for large volume enrichment cultures and containing 11-fold concentration of each ingredient was filter sterilized with a GV Durapore membrane filter (0.22 μm) to prevent burning. TTC (2,3,5-triphenyl tetrazolium chloride) was added to a final concentration of 0.1% in tryptone agar plates (1.2% agar) and used for spot confirmation and plaque assay (Yanko *et al.* 1999). Water samples (1 l) were inoculated with 10 ml of host bacterial culture [*E. coli* HS (pFamp)R] and 100 ml of 11X tryptone broth with antibiotics (ampicillin–streptomycin). After a 48-hour period of incubation at 35°C, 10 μl of the enriched sample was then spotted onto a lawn of host bacteria, incubated at 37°C and examined for lysis zones. Results were expressed as presence/absence. To determine if the isolates were F + DNA or F + RNA coliphage, RNAse was incorporated in the plating growth medium. One plate contained RNAse and one did not. Aliquots of 5–10 μl were spotted onto a lawn of host bacteria, incubated at 37°C, and examined for lysis zones. The coliphage that were inhibited from plaquing were considered F + RNA coliphage (Yanko *et al.* 1999).

Waterborne pathogens

Large-volume sample analysis (>100 l) via cartridge filtration was used for protozoa (*Cryptosporidium* and *Giardia*) and infectious enteric viruses. For protozoa, water samples were filtered through a 254 mm-long polypropylene yarn-wound cartridge filter (1.0 μm, nominal porosity, Filterite, Timonium, Maryland) using a gasoline-powered portable water pump with flow rates maintained at 4–11 l min^{-1}. For enteric viruses a 0.2 μm, positively charged zeta pleated cartridge filter (1 MDS, Cuno Inc., Meriden, Connecticut) was used. After collection, the filters were placed on ice and transported to the laboratory for processing.

The occurrence of *Cryptosporidium* oocysts and *Giardia* cysts was determined by the immunofluorescence microscopy technique according to a modified version of the United States Environmental Protection Agency Information Collection Rule method (USEPA/ICR) (USEPA 1995*a*). The modification included the use of the Dynal immunomagnetic separation (IMS) technique for selective separation of (oo)cysts from other debris and the inclusion of 4′,6′-diamidino-2-phenylindole (DAPI) for confirmation of waterborne (oo)cysts.

The isolation and detection of enteric viruses was carried out by the production of cytopathogenic effects (CPE) in cell culture. Initially, water samples were concentrated by filtration and organic flocculation procedures (USEPA 1995*b*). Water sample concentrates were filter sterilized and inoculated onto Buffalo Green Monkey (BGM), Rhabdosarcoma and MA-104 cells. Cytopathogenic effects (CPE) indicated the presence of enteric viruses in the water samples analysed. Infectious enteric viruses were expressed as most probable number per 100 l (MPN per

Table 1 | Microbial indicators in proposed water sources for restoration

	Water type	
Indicator	**Storm water**	**Reclaimed water**
TC (CFU $100\,ml^{-1}$)		
Percentage positive	100% (4/4)	75% (3/4)
Maximum value	2.35×10^4	10
Minimum value	1.2×10^3	<0.058
Arithmetic mean	1.2×10^4	8
Geometric mean	6.59×10^3	7
FC (CFU $100\,ml^{-1}$)		
Percentage positive	100% (4/4)	75% (3/4)
Maximum value	1.55×10^3	1
Minimum value	7.0×10^1	0.45
Arithmetic mean	8.73×10^2	0.82
Geometric mean	5.25×10^2	0.52
E (CFU $100\,ml^{-1}$)		
Percentage positive	100% (4/4)	0% (0/4)
Maximum value	3.35×10^3	<0.8
Minimum value	3.0×10^1	<0.058
Arithmetic mean	1.90×10^3	NA[a]
Geometric mean	8.08×10^2	NA
C. perfringens (CFU $100\,ml^{-1}$)		
Percentage positive	100% (4/4)	25% (1/4)
Maximum value	3.0×10^1	10
Minimum value	9.3×10^0	<0.058
Arithmetic mean	2.1×10^1	NA
Geometric mean	1.92×10^1	NA

[a]Not applicable.

100 l) taking into account the equivalent volume examined (USEPA 1995*b*).

Statistical analysis

Arithmetic averages were calculated at each site for each variable. Values less than the limit of detection were considered as zero in these calculations. For those sites where all values were less than the limit of detection, the individual limits of detection for each sample were averaged. The differences in the limits of sensitivity for different samples reflect differences in sample volumes. The biological data were transformed to $\log_{10}$ data ($\log_{10}$ of $Y + 1$) and geometric averages were calculated. For those samples that generated microbial counts (lakes, storm water and reclaimed effluents) a statistical test (Spearman rank order test) was performed to determine the relationship among concentrations of the various indicators. Because the total coliform, fecal coliform, enterococci and *C. perfringens* data set did not follow a normal distribution, correlations between counts were evaluated using the Spearman rank order test. This nonparametric test measures the strength of association between pairs of variables without specifying which variable is dependent or independent and assumes that error distributions in the compared data set are the same.

RESULTS

Bacterial indicators

Arithmetic and geometric means for levels of each bacterial indicator found in the source waters for restoration and ambient waters within the wetland, respectively, are given in Tables 1 and 2.

High levels of bacterial indicators were detected in storm water samples. The arithmetic means for total and fecal coliforms (TC and FC, respectively), enterococci (E) and *Clostridium perfringens* were: 1.2×10^4 CFU $100\,ml^{-1}$ (TC), 8.73×10^2 CFU $100\,ml^{-1}$ (FC), 1.90×10^3 CFU $100\,ml^{-1}$ (E) and 2.1×10^1 CFU $100\,ml^{-1}$ (*C. perfringens*). Bacteriophages were detected by the agar overlay method in two of four storm water samples and levels ranged from 40

Table 2 | Microbial indicators in water samples collected in four major lakes within the wetland

	Lake			
Indicator	**A**	**B**	**C**	**D**
TC (CFU $100\,ml^{-1}$)				
Percentage positive	100% (4/4)	100% (2/2)	100% (4/4)	100% (3/3)
Maximum value	4.75×10^4	7×10^3	6.45×10^3	3.9×10^3
Minimum value	8.0×10^1	1.6×10^3	2.59×10^2	3.58×10^2
Arithmetic mean	1.27×10^4	4.3×10^3	1.89×10^3	1.95×10^3
Geometric mean	1.65×10^3	3.3×10^3	7.45×10^2	131×10^3
FC (CFU $100\,ml^{-1}$)				
Percentage positive	100% (4/4)	100% (2/2)	100% (4/4)	100% (3/3)
Maximum value	1.95×10^4	4.75×10^4	6.0×10^2	4.05×10^3
Minimum value	2.3×10^1	3.87×10^1	2.5×10^1	2.63×10^2
Arithmetic mean	2.96×10^3	2.25×10^3	2.74×10^2	1.64×10^3
Geometric mean	4.36×10^2	1.3×10^3	1.68×10^2	8.61×10^2
E (CFU $100\,ml^{-1}$)				
Percentage positive	50% (2/4)	100% (2/2)	100% (4/4)	66% (2/3)
Maximum value	4.75×10^4	2.20×10^3	4.0×10^1	8.8×10^1
Minimum value	<1	4.5×10^1	4×10^0	<1
Arithmetic mean	5.26×10^1	1.12×10^3	1.95×10^1	5.6×10^1
Geometric mean	5.4×10^0	3.15×10^2	1.42×10^1	1.02×10^2
C. perfringens (CFU $100\,ml^{-1}$)				
Percentage positive	75% (3/4)	0% (0/2)	100% (4/4)	100% (3/3)
Maximum value	1.7×10^1	<1.3	6.0×10^1	9.2×10^1
Minimum value	<0.45	<0.45	6.0×10^0	8.0×10^0
Arithmetic mean	9.54×10^0	<0.875	2.3×10^1	3.9×10^1
Geometric mean	8.3×10^0	<0.7649	1.4×10^1	2.4×10^1

to 121 PFU 100 ml^{-1}. These results were obtained using *E. coli* host bacterial culture ATCC [American Type Culture Collection] 15597, which enables the detection of somatic coliphage. The enrichment protocol and the bacterial host *E. coli* HS (pFampR) enabled the detection of F-specific RNA coliphage in three of the four storm water sampling events.

The arithmetic means for total and fecal coliform in reclaimed water samples collected were 8 CFU 100 ml^{-1} and 0.82 CFU 100 ml^{-1}, respectively. Enterococci were not detected and *C. perfringens* was present in one sample at a level of 10 CFU 100 ml^{-1}. Bacteriophages were not detected in these samples.

Among the sampled lakes, Lake A had the greatest mean concentration of bacterial indicators (Table 2).

The agar overlay method and the *E. coli* host bacterial culture (ATCC 15597) allowed the detection and enumeration of bacteriophages in Lake B and the level was 5 PFU 100 ml^{-1}. When the bacterial host *E. coli* HS (pFamp)R and the agar overlay method were used no F-specific coliphage were detected in the lakes. However, the presence/absence test based on the enrichment protocol enabled the detection of F-specific coliphage in some of the sampled lakes that were negative by the agar overlay method. Lakes A and C were positive for bacteriophages in one out of four samples collected at each site using the enrichment protocol. This is an indication of very low levels of F- coliphage. F-specific coliphage in this sample were found to be RNA bacteriophages using the method described by Yanko *et al.* (1999).

Bacterial indicators were not detected in any of the sampled wells in a total filtered volume of 1,750 ml (data not shown). However, one of the water wells was positive for FRNA coliphage using the enrichment protocol.

Protozoa (*Giardia* and *Cryptosporidium*) and infectious enteric viruses

The level of protozoan parasites and enteric viruses varied among the water samples tested (Table 3). The results demonstrated that the highest level of *Cryptosporidium* oocysts and infectious enteric viruses were present in storm water and lakes. In contrast, the occurrence of waterborne pathogens in the reclaimed effluent was relatively low. Pathogen occurrence in groundwater was intermittent (Table 3).

DISCUSSION

There are few or no criteria governing water quality in wetlands or non-contact recreational waters used for recharge to groundwater wells. Background water quality in the lakes in this Florida wetland and potential recharge waters were compared with Florida standards.

The water quality data obtained from the various waters were initially assessed against the regulatory standards of the State of Florida Class III surface water standards for freshwater according to Chapter 62-302.530 FAC (Florida Administrative Code, Criteria for Surface Water Quality Classifications). Total and fecal coliforms are expressed in two tiers in recognition of the fact that sampling will produce a range in results. The total and fecal coliform standards currently used in the state of Florida are:

- 1,000 CFU 100 ml^{-1} as a monthly average (geometric mean); nor exceed 1,000 CFU 100 ml^{-1} in more than 20% of the samples examined during any month; $\leq$2,400 CFU 100 ml^{-1} at any time (total coliforms);
- No one sample can exceed 800 CFU 100 ml^{-1}, no more than 10% of samples can exceed 400 CFU 100 ml^{-1}, and the geometric mean of ten samples collected within 30 days cannot exceed 200 CFU 100 ml^{-1} (fecal coliforms).

Lake A is used for non-contact recreational water activities. The maximum total (2,400 CFU 100 ml^{-1}) and fecal coliform (800 CFU 100 ml^{-1}) limit was exceeded in Lakes A, B and D and in storm water samples collected from the drainage Canal (Figures 1 and 2). USEPA (1986) has established a one-time sample threshold for enterococci of 104 CFU 100 ml^{-1}. Thus the guidance threshold was exceeded in two of the lakes.

Currently, there are no established thresholds or recommended limits for bacteriophages. Although somatic coliphage may not be found exclusively in human fecal waste, their presence has been used as an indication of the extent of general contamination in an area. A level of 100 PFU 100 ml^{-1} based on previous research in our laboratory has been proposed as a guidance level for

Table 3 | Number of protozoan parasites and enteric viruses in environmental waters

	Cryptosporidium		*Giardia*		Enteric viruses	
Water type	**No. samples positive (%)**	**Average oocysts per 100 l (range)**	**No. samples positive (%)**	**Average cysts per 100 l (range)**	**No. samples positive (%)**	**MPN 100 l^{-1}**
Storm water	1 of 4 (25)	72 ± 144 (<2–287)	0 of 4 (0)	–[a]	4 of 4 (100)	2 ± 2 (0.48–4.4)
Reclaimed water	2 of 4 (25)	2 ± 3 (<0.24–6.5)	0 of 4 (0)	–	1 of 4 (25)	0.02 ± 0.04 (<0.08–0.09)
Lakes						
A	1 of 2 (50)	2 ± 3 (<2–4)	0 of 2 (0)	–	1 of 2 (50)	0.8 ± 1 (<0.2–1.7)
B	3 of 3 (100)	40 ± 63 (2–113)	1 of 3 (33)	1 ± 2 (<0.9–4)	1 of 3 (33)	0.2 ± 0.4 (<0.3–0.6)
C	1 of 2 (50)	10 ± 15 (<1–21)	0 of 2 (0)	–	1 of 2 (33)	0.2 ± 0.4 (<0.02–0.6)
D	2 of 2 (100)	3 ± 2 (2–4)	0 of 2 (0)	–	2 of 2 (100)	1.4 ± 0.6 (0.96–1.82)
Wells						
1	0 of 2 (0)	–	1 of 2 (50)	0.7 ± 1 (<0.2–1.5)	1 of 2 (50)	0.07 ± 0.1 (<0.06–0.14)
2	2 of 2 (100)	0.3 ± 0.3 (0.1–0.53)	0 of 2 (0)	–	0 of 2 (0)	–
3	0 of 2 (0)	–	0 of 2 (0)	–	2 of 2 (100)	0.11 ± 0.05 (0.07–0.15)
4	0 of 5 (0)	–	0 of 5 (0)	–	1 of 5 (20)	0.02 ± 0.05 (<0.02–0.12)

[a]none detected.

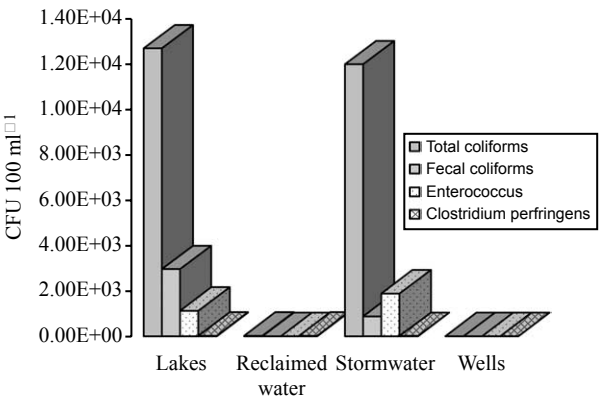

Figure 1 | Levels of microbial indicators in ambient (Lakes and Wells) and proposed source waters for restoration (Reclaimed water and Stormwater).

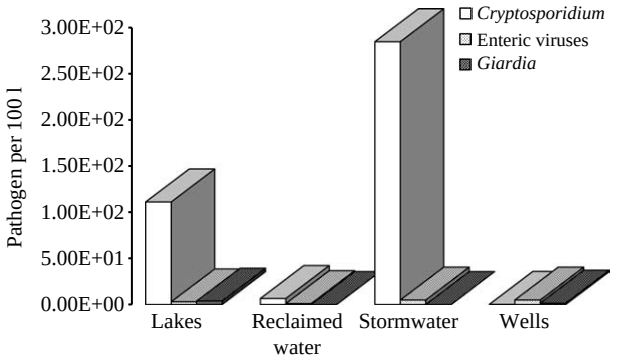

Figure 3 | Level of enteric pathogens isolated from ambient waters (Lakes and Wells) and source waters proposed for restoration (Reclaimed water and Stormwater).

evaluation of water quality (Lipp *et al.* 2001). In the present study, this level was exceeded in one storm water sample collected from the drainage area (120 PFU $100\,ml^{-1}$). This was also the site with the highest level of indicator organisms and waterborne pathogens (Figure 3). This situation is expected since fecal droppings of pets (dogs, cats), birds and other animals that occur on land can be washed into lakes and storm drains by rain, increasing the concentrations of fecal indicators and enteric pathogens in environmental waters (Roll & Fujioka 1997).

The bacterium *Clostridium perfringens* has been suggested as an indicator of sewage pollution. Based on work done in Hawaiian streams, Fujioka & Shizumura (1985) recommended a guideline of 50 CFU $100\,ml^{-1}$ for fresh waters. This recommended threshold was exceeded in Lakes C and D, but not in Lake A. Similar levels of this bacterium have been reported in other areas of Florida (Lipp 1999). However, the results of this and other investigations (Rose *et al.* 2000) have demonstrated that *C. perfringens*, while indicative of fecal pollution, only has limited value as an alternative indicator in areas of large dilution because of its low concentration in feces. No differences in levels of *C. perfringens* were seen between the background waters (lakes) and storm waters.

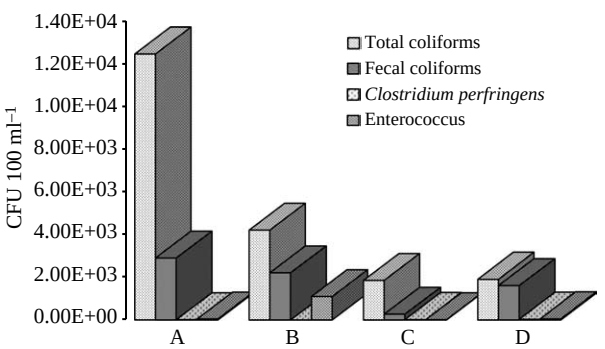

Figure 2 | Levels of water quality indicator bacteria in lakes within the wetland (A, B, C and D correspond to lake designations as described in the text).

Correlations between microbial indicator counts obtained in this study were evaluated using the Spearman rank order test. Significant correlations were found using the Spearman rank order correlation coefficient r_s between total coliforms and fecal coliform (0.91, $p < 0.0001$), which was to be expected, between total coliforms and enterococci (0.66, $p < 0.001$), and fecal coliforms and enterococci (0.61, $p < 0.05$). *C. perfringens* and bacteriophages did not show any statistical correlation with the rest of the microbial indicators.

This investigation, like previous investigations demonstrated that the presence of enteric pathogens was not significantly correlated with levels of indicators in surface waters (Rose *et al.* 1988; Callahan *et al.* 2001; Lipp *et al.* 2001). Therefore, in order to evaluate the true evidence of fecal contamination, risk of exposure to contaminated water and potential health impacts, the assessment of *Cryptosporidium*, *Giardia* and infectious enteric viruses is the preferred approach.

Marine surface waters in southern Florida have been analysed for protozoan parasites and enteroviruses; the levels found in those studies (Lipp 1999; Rose *et al.* 2000; Callahan *et al.* 2001) were relatively low compared with levels found in the present investigation of freshwaters.

Groundwater samples were negative for the current microbial indicators except one water well, which was positive for FRNA coliphage. The enrichment protocol and the bacterial host used (*E. coli* strain HS(pFamp)R) enabled the detection of a low number of these phages. Under laboratory testing conditions up to 1 PFU of FRNA coliphage added to 1,000 l has been detected (Fujioka & Yoneyama 2001); therefore this technique is sensitive enough to detect low levels of bacteriophages.

Monitoring groundwater samples for several potential microbial fecal indicators, including FRNA coliphage, is currently used to characterize the quality of the water with respect to vulnerability to contamination (USEPA 1999; Fujioka & Yoneyama 2001). Yanko *et al.* (1999) have suggested the uses of FRNA coliphage as tracers to assess the potential for virus migration in soil. Waterborne pathogens such as *Cryptosporidium*, *Giardia* and enteric viruses were also detected in these sites indicating groundwater vulnerability to fecal contamination.

Virus occurrence in the water wells was intermittent. Enteric viruses are species specific so that human health risks are only significantly associated with viruses from human fecal sources. The methodologies employed tend to favour detection of human enteroviruses; however the cell lines can possibly pick up other mammalian viruses, for example viruses from cows which would not represent a human health risk. Perhaps viruses from horses could be detected, which may be a potential source in the study site. There was no positive identification of the virus types for the restoration project; therefore the viruses cannot be definitively described.

The presence of *Cryptosporidium* in the water wells also suggests some vulnerability. Studies on groundwater in the US (once thought to be a more protected source) reported that between 9.5% and 22% of samples were positive for *Cryptosporidium* (Hancock *et al.* 1998, Moulton-Hancock *et al.* 2000). In the present study, *Giardia* and *Cryptosporidium* were detected once at two water wells and these wells were taken out of service. To date, additional testing has been carried out in four active water wells to determine whether groundwater sources within the wetland are under direct influence from surface water systems (lakes and wetlands). The testing is based on the Consensus Method (Microscopic Particulate Analysis) for Determining Ground Waters Under the Direct Influence of Surface Water (USEPA 2003). The preliminary results have demonstrated that the water wells have either no risk or moderate risk of surface water contamination.

Impacts on groundwater by viruses are of great concern because of the resistant nature of the viral structure and the colloidal size (20 nm), which makes this group of microorganisms easily transported through soil systems (Gerba & Bitton 1984). Viruses can also survive several months in groundwater and are more resistant to water disinfection than are the coliforms (Gerba & Rose 1990; Yates & Yates 1988). Studies in the United States have found viruses in 20% to 30% of the groundwater where coliforms were not predictive of viral contamination (Gerba and Rose 1990). New techniques using polymerase chain reaction (PCR) have shown that there is much more viral occurrence in groundwater than previously recognized. This has led to disease in non-disinfected systems (Borchardt *et al.* 2003).

The general hydrology of the study site consists of the surficial aquifer and the Upper Floridan aquifer separated by a clay semi-confining unit. The water wells of this area are approximately 122 to 183 m deep and withdraw water from the Upper Floridan aquifer. Several investigations have demonstrated that microbial contaminants may enter the subsurface directly via structures which by-pass the soil zone such as septic tanks. There are four septic tank systems operating within the wetland. *Cryptosporidium* oocysts are spherical particles 4 to 6 μm in diameter, with a specific gravity of 1.07 g cm^{-3} (Current 1987). Like many microbes, *Cryptosporidium* oocysts have been classified as colloidal particles. Recent investigations have shown that *Cryptosporidium* oocysts entrained in surface or subsurface water will travel at the same velocity as the water (Brush *et al.* 1999). An understanding of the mechanisms governing waterborne transport of oocysts is needed to develop management practices that reduce or eliminate oocysts migration from the source of contamination (fecal deposition in the soil surface or structures which by-pass the soil zone such as septic tanks) and to facilitate assessment of the aquifer's and individual well's vulnerability to contamination with oocysts.

Cryptosporidium oocysts have been proven to remain viable for up to 16 months (approximately 480 days) when stored in water at 4°C (with antibiotics added) (Fayer *et al.*

1998; Fayer *et al.* 2000). As temperatures approach 20–25°C, a temperature that would be expected in the lakes at the study area, viability decreases. The average temperature in groundwater at the study area was estimated to be 22°C and the surrounding surface waters to be 25°C (sampling data). At these temperatures the estimated die-off rates are 0.03944 and 0.0589 natural log per day (Walker & Stedinger 1999). During the rainy summer months, the surface water temperatures can be as high as 33°C, increasing the die-off rate during this time period.

The hydrologic modelling results have shown that the first arrival of restoration source water to the water wells is 175 days. Based on the decay function developed by Walker & Stedinger (1999) potential oocyst viability was examined in this system. The rate constant (0.03944 natural log per day) was used to predict the decrease in the concentration of viable *Cryptosporidium* oocysts over time, which in turn was related to distance of the water flow velocity. Using a first order decay function an estimate of the reduction of oocysts can be calculated: $C = C_o e - \lambda t$, where C is equal to the concentration in groundwater at time t, C_o corresponds to initial concentration, λ is equal to die-off rate and t corresponds to time. Assuming that the oocyst levels found in the storm water sample (287 per 100 l, average 72 per 100 l) were viable/infectious, and that the drainage canal was used to restore the lakes, then the oocysts in groundwater would be reduced to approximately 0.3 viable/infectious oocysts per 100 l after 175 days. Therefore the numbers of viable oocysts would be reduced by almost 1,000-fold owing to die-off during subsurface transport.

Yates & Yates (1988) proposed a model to estimate inactivation of viruses in soils using the coliphage MS2 as a standard (Log_{10} inactivation $= -0.181 + 0.0214 \times T$°C). The observed water temperature at the study site averaged 22°C. Using the observed groundwater temperature, 22°C, the inactivation rate using the model is 0.289 $\log_{10}$ per day. Bitton *et al.* (1983) estimated a Polio 1 virus inactivation rate in Florida groundwater of 0.0456 $\log_{10}$ per day. The equation derived from this study was: $y = 0.0019X + 4.84$, where the decay rate (k) estimated from the slope of the linear regression of the log transformed data was 0.0019 h^{-1} ($r^2 = 0.99$). The levels of virus reductions were calculated using these inactivation rates. Based on the hydrologic modelling results, the reductions of viruses would be 7.98 $\log_{10}$ reductions based on the rate described by Bitton *et al.* (1983).

In summary, both viruses and viable *Cryptosporidium* numbers will be reduced in the environment. These microorganisms will die off over time, and numbers of viable organisms will be reduced by approximately 100,000,000 and 1,000-fold, respectively, after 175 days, which is the model-predicted time that it takes the restoration water to migrate to the first water well. In addition, physical removal through adsorption is estimated for viruses at a minimum of 99%. Oocyst removal through filtration will also take place, but was not quantified in this study.

Model-derived travel times to the water wells and dilution factors can be used in the exposure assessment for microbial risks. However, the limited water quality sampling results to date suggest that there may be some low, background level of contaminating microorganisms in the groundwater resulting in an ambient level risk of exposure to pathogens. This may suggest that the wells themselves need to be refurbished. The two *Cryptosporidium* oocysts found in well 2 suggest vulnerability as mentioned above, even if these oocysts are not viable or a strain that is not infectious to humans. Depending on the average concentration of oocysts in groundwaters, this ambient risk could outweigh future risks due to the small percentage of source waters (3.3%) migrating to the water wells. Owing to the limited number of samples and uncertainties in computing average *Cryptosporidium* concentrations, additional characterization of the ambient water quality is necessary prior to completing the microbial risk assessment.

This study provided information on well vulnerability and risk that would have not been assessed without appropriate pathogen monitoring. These data can lead to better well construction corrections and maintenance as well as assessment of the aquifer.

The results of the microbiological monitoring programme indicate that, between the two proposed source waters for restoration, the storm water source had the highest detection of microbial indicators and pathogens. Therefore, the potential use of storm water as a source for restoration will depend on further characterization of *Cryptosporidium* levels using more efficient concentration techniques because *Cryptosporidium* oocysts are more

stable during transport to water wells (Rose *et al.* 1996). In addition, the identification of predominant species and the assessment of the viability/infectivity of isolated oocysts along with identification of predominant viruses present in storm water sources will help to refine the microbial risk assessment.

The acceptability of reclaimed water for any particular use is dependent on the physical, chemical and microbiological quality of the water. Factors that affect the quality of reclaimed water include source water quality, wastewater treatment processes and treatment effectiveness, treatment reliability, and distribution system design and operation (Crook & Surampalli 1996). The presence of pathogenic microorganisms in wastewater creates the potential for adverse health effects where there is contact, inhalation or ingestion of microbiological constituents of health concern. The criteria established for the discharge of reclaimed water to and from receiving wetlands (rule 62–611, Florida Administrative Code) do not specify any guidelines for microbiological constituents of health concern. Therefore, the recommended threshold established for reuse activities in the state of Florida was used to evaluate the adequacy of reclaimed water for wetland and lake restoration. The threshold limits for reuse activities were all met. However, low levels of enteric viruses and protozoan parasites were found in these samples.

Reclaimed water would be a more consistent and reliable source for restoration compared with storm water in both quantity and quality. In addition, any further concerns regarding health risks could be monitored for and treated prior to restoration. Ongoing monitoring of groundwater and ambient waters is also recommended.

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