APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2005, p. 3163–3170 Vol. 71, No. 6
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.6.3163–3170.2005

Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection†

Valerie J. Harwood,[1]* Audrey D. Levine,[2] Troy M. Scott,[3] Vasanta Chivukula,[1] Jerzy Lukasik,[3] Samuel R. Farrah,[4] and Joan B. Rose[5]

Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620[1]; Department of Civil and Environmental Engineering, ENB 118, University of South Florida, 4202 E. Fowler Ave., Tampa, Florida 33620[2]; Biological Consulting Services of N. Florida, Inc., 4641 N.W. 6th Street, Suite A, Gainesville, Florida 32609[3]; Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611[4]; and Department of Fisheries and Wildlife and Crop and Soil Sciences, 13 Natural Resources Building, Michigan State University, East Lansing, Michigan 48824[5]

Received 27 September 2004/Accepted 20 December 2004

The validity of using indicator organisms (total and fecal coliforms, enterococci, *Clostridium perfringens*, and F-specific coliphages) to predict the presence or absence of pathogens (infectious enteric viruses, *Cryptosporidium*, and *Giardia*) was tested at six wastewater reclamation facilities. Multiple samplings conducted at each facility over a 1-year period. Larger sample volumes for indicators (0.2 to 0.4 liters) and pathogens (30 to 100 liters) resulted in more sensitive detection limits than are typical of routine monitoring. Microorganisms were detected in disinfected effluent samples at the following frequencies: total coliforms, 63%; fecal coliforms, 27%; enterococci, 27%; *C. perfringens*, 61%; F-specific coliphages, ~40%; and enteric viruses, 31%. *Cryptosporidium* oocysts and *Giardia* cysts were detected in 70% and 80%, respectively, of reclaimed water samples. Viable *Cryptosporidium*, based on cell culture infectivity assays, was detected in 20% of the reclaimed water samples. No strong correlation was found for any indicator-pathogen combination. When data for all indicators were tested using discriminant analysis, the presence/absence patterns for *Giardia* cysts, *Cryptosporidium* oocysts, infectious *Cryptosporidium*, and infectious enteric viruses were predicted for over 71% of disinfected effluents. The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed. Monitoring a suite of indicator organisms in reclaimed effluent is more likely to be predictive of the presence of certain pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested by this study.

Reclaimed water is derived from treated municipal wastewater. The treatment processes used for production of reclaimed water provide multiple barriers (biological treatment, physical removal, and chemical disinfection) for control of pathogens. Reclaimed water is used for nonpotable applications such as irrigation, cooling water, industrial process water, and environmental enhancement (17). Indirect potable reuse occurs through groundwater recharge or surface water replenishment, and is assuming greater importance with increased production of reclaimed water. As water use in the United States (7) and worldwide increases, the importance of reclaimed water to sustainable water resources will continue to increase (17).

A major goal of wastewater reclamation facilities is to reduce pathogen loads in order to decrease public health risks associated with exposure. The effectiveness of pathogen control is indirectly assessed through routine monitoring of the reclaimed water (final effluent) by using grab samples to detect standard indicator bacteria such as total or fecal coliforms. Treatment practices for production of reclaimed water vary depending on the ultimate intended use(s) of the water and local regulatory requirements. Currently, there are no universal standards governing the production and quality of reclaimed water, although the World Health Organization has developed guidelines for the use of reclaimed water (35) that recommend monitoring fecal coliforms and intestinal nematodes. In the United States, there are no federal standards controlling the quality of reclaimed water, and individual states have developed guidelines or implemented specific treatment and monitoring requirements that are intended to protect the public from exposure to pathogens. Due to the inherent constraints associated with pathogen monitoring, indicator organisms are employed as surrogates for pathogens. In some states, total coliform bacteria are used as the indicator organism (6); however, in the majority of states that have specific regulations, the microbiological safety of reclaimed water is evaluated by daily monitoring of fecal coliform bacteria in the disinfected effluent based on a single, 100-ml grab sample (3). In addition, periodic monitoring of viruses and/or protozoan pathogens has been required by a few states, including Arizona, California, and Florida (3).

It has been widely demonstrated that coliform bacteria do not adequately reflect the occurrence of pathogens in disinfected wastewater effluent due to their relatively high susceptibility to chemical disinfection (18) and failure to correlate

* Corresponding author. Mailing address: Department of Biology, SCA 110, University of South Florida, 4202 E. Fowler Ave., Tampa, FL 33620. Phone: (813) 974-1524. Fax: (813) 974-3263. E-mail: vharwood@cas.usf.edu.

† Florida Agricultural Experiment Station Journal Series no. R10718.

TABLE 1. Comparison of wastewater reclamation facilities sampled for indicator organisms and pathogens in this study

Facility	Avg capacity ($m^3 \cdot s^{-1}$)	Biological treatment	Chemical use prior to filtration	Filter composition (depth, m)	Backwash frequency (h)	Type of disinfection
A	0.04	Activated sludge	None	Fabric (0.02)	24 to 72	Chloramines[a]
B	0.4	Activated sludge	Chlorine	Sand (0.3)	Automatic (daily)	Chloramines[a]
C	0.4	Activated sludge	Cationic polyelectrolyte	Anthracite (1.2)	48	Chloramines[a]
D	0.7	Activated sludge	None	Anthracite (0.8), sand (0.25)	48 to 168	Chloramines
E	0.08	Nitrification	None	Sand (1.2), upflow	Continuous	Ultraviolet light
F	0.13	Biological nutrient removal	None (alum added to secondary clarifier)	Anthracite (0.6), sand (1.2)	48 to 168	Chlorine

[a] Chloramines are formed due to the reaction of chlorine with residual ammonia.

with protozoan parasites such as *Cryptosporidium* (5) and enteric viruses (13). Alternative microbiological indicators have been suggested for evaluation of wastewater, drinking water, and environmental waters, including *Enterococcus* spp. (18), *Clostridium perfringens* (9, 20), and coliphages (8, 10, 20).

To date, there have been only a few studies of reclaimed water in which the levels of indicator organisms have been directly compared to those of viral, bacterial, or protozoan pathogens at each stage of treatment (23, 24). In this work, the validity of using coliform bacteria and alternative microbial indicators to predict the presence or absence of pathogens, and thus to assess the public health risk, was evaluated using disinfected effluent from six wastewater reclamation facilities in the United States. The facilities varied in location (Arizona, California, and Florida), size, and treatment practices and were each sampled at least five times over a 1-year period. Each sample was analyzed for a suite of indicator bacteria, coliphages, enteric viruses, and protozoan pathogens, and predictive relationships among the microbial groups were evaluated by several statistical methods, including binary logistic regression and discriminant analysis (DA).

MATERIALS AND METHODS

Facilities. Six wastewater reclamation facilities in the United States were each sampled at least five times over a 1-year period. A comparison of the treatment characteristics is given in Table 1. The facilities represent a cross-section of typical treatment approaches that are used for production of reclaimed water.

Sampling. All samples were aseptically collected in sterile containers (or sterile filters). Samples were immediately placed on ice in coolers and were kept on ice until processed. At each facility, samples were collected from the influent (untreated wastewater), secondary clarifier (biological treatment), filtered effluent, and disinfected effluent (reclaimed water). Samples were collected under peak flow conditions to provide a "worst-case" scenario for each facility. Each facility was sampled approximately bimonthly over a 1-year period, resulting in at least five samplings per facility.

Sample volumes collected for bacterial enumeration were 50 ml of influent, 500 ml from the secondary clarifier, 2 liters of effluent from the filtration stage, and 2 liters of disinfected effluent. Assays were performed in triplicate. Large volumes (up to 53 liters) were filtered for protozoan parasite and virus assays. Detection limits for bacterial indicators in disinfected effluent were 0.2 to 0.6 CFU $\cdot$ 100 ml^{-1}, those for coliphages were 10 PFU $\cdot$ 100 ml^{-1}, those for enteric viruses were 0.3 to 1.4 most probable number (MPN) $\cdot$ 100 $liters^{-1}$, those for *Cryptosporidium* oocysts were 2.0 to 6.9 oocysts $\cdot$ 100 $liters^{-1}$, those for infectious *Cryptosporidium* were 0.29 to 4.1 MPN $\cdot$ 100 $liters^{-1}$, and those for *Giardia* were 1.8 to 5.2 cysts $\cdot$ 100 $liters^{-1}$.

Bacterial enumeration. Indicator bacteria were quantified by membrane filtration using 47-mm cellulose acetate filters with a nominal pore size of 0.45 μm. Total coliform bacteria were cultured on mEndo LES agar (Difco, Sparks, MD) for 24 h at 37°C. Colonies that produced a green sheen were enumerated as total coliforms (2). Fecal coliform bacteria were cultured on mFC agar (Difco, Sparks, MD) for 24 h at 44.5°C in a water bath. Blue colonies were enumerated as fecal coliforms (2). *Escherichia coli* (ATCC 9637) was used as the positive control for all coliform measurements. Enterococci were cultured on mEI agar (31, 32). Plates were incubated at 41°C for 24 h, and colonies with a blue halo were enumerated as enterococci. *Enterococcus faecalis* (ATCC 19433) was used as a positive control. *Clostridium perfringens* was isolated on mCP agar (Acumedia Manufacturers, Inc.) (4). Plates were transferred to gas pack bags (BBL GasPak; Becton Dickinson) and sealed. After 24 h of incubation at 45°C, colonies were exposed to ammonium hydroxide fumes. All of the yellow/straw-colored colonies that turned pink/magenta were counted. *C. perfringens* (ATCC 13124) was used as a positive control.

Bacteriophage analysis. Coliphages were analyzed by the agar overlay method of Adams (1). Two *E. coli* host strains were used in separate assays: *E. coli* HS (pFamp) R (ATCC 700891), which infects male-specific (F^+) coliphages very efficiently and somatic coliphages poorly (8), and *E. coli* C3000 (ATCC 15597), which should host both somatic and F^+ coliphages (14). Serial dilutions of samples were made in phosphate-buffered saline according to expected phage concentrations at each treatment step. Five replicate volumes of 0.1 ml to 2 ml were plated for each dilution, except in the case of the disinfected effluent samples, for which 10 replicates of 2 ml each were plated. PFU $\cdot$ 100 ml^{-1} were calculated after 24 h of incubation (2).

Enteric viruses. The U.S. Environmental Protection Agency (EPA) methodology (30) was used for the detection of enteric viruses. Influent sample volumes were based on the amount of water that could be processed without clogging the filter. Typically less than 100 liters was filtered for each influent sample, depending on water quality (i.e., content of suspended solids). Larger sample volumes were used for the other sample locations, i.e., ~190-liter samples from the secondary clarifiers and ~380-liter samples from the filtration and disinfection processes. Water samples were pumped through Virusorb 1MDS filters (Cuno, Inc.), which were eluted with 1 liter of 1.5% beef extract (BBL V) in 0.05 M glycine (pH 9.5, ~25°C) (U.S. EPA/ICR). The eluted sample was concentrated by organic flocculation and assayed for enterovirus by the observation of cytopathic effects (CPE) on recently passed (<4 days) cell lines. Three cell lines, i.e., buffalo green monkey, rhabdosarcoma (ATCC CCL-136), and MA-104 (ATCC CRL-2378.1) cells, were used for this purpose. Positive controls were processed in a separate room, using poliovirus I. CPE on each cell line were observed, and the most dilute sample showing CPE was recorded. MPN determinations were performed using EPA-released software (Most Probable Number Calculator version 4.04; http://www.epa.gov/microbes/other.htm).

Protozoa. For the detection of *Giardia* and *Cryptosporidium*, samples were concentrated by filtration using Gelman Envirochek HV cartridge filters and processed according to the manufacturer's instructions. Following filtration, samples were processed by immunomagnetic separation (Dynal, Inc.) and immunofluorescent antibody detection (Easy Stain; Biotech Frontier, Australia) according to the procedure outlined in U.S. EPA method 1623 (33). Sample volumes varied depending upon the treatment stage and the amount of water that could be filtered, i.e., 0.5 to 1.0 liters influent, ~19 liters secondary effluent, ~38 liters effluent from filters, and up to 53 liters disinfected effluent. Detection limits varied with the total volume of sample filtered and processed. Each concentrated sample was divided into two aliquots: one for cell culture viability testing and the other for microscopic enumeration. Equivalent volumes were calculated, and the results were reported as cysts or oocysts $\cdot$ 100 $liters^{-1}$.

***Cryptosporidium* infectivity.** Concentrates from the immunomagnetic separation procedure were inoculated onto HCT-8 cell monolayers in eight-well chamber glass cell culture slides. The cultures were incubated in a 5% CO_2 atmosphere at 37°C for 48 h. Infective *Cryptosporidium* was enumerated by the focus detection method-MPN assay (27). Results were reported as infectious oocysts $\cdot$ 100 $liters^{-1}$.

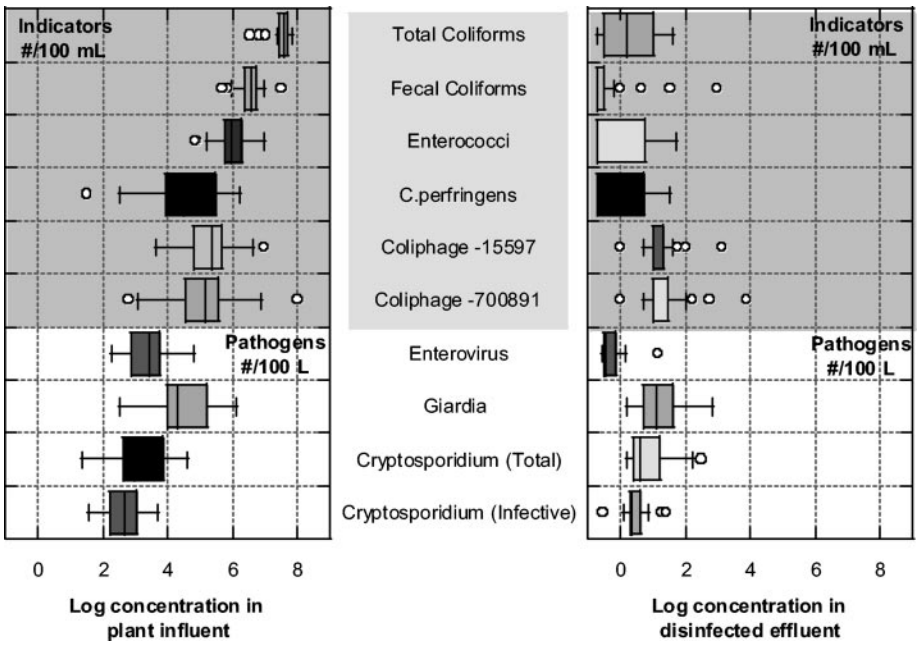

FIG. 1. Mean indicator organism and pathogen concentrations in untreated wastewater and disinfected effluent from six wastewater reclamation facilities ($n = 30$). Log_{10} concentrations of bacterial indicators (CFU · 100 ml^{-1}), coliphages on *E. coli* 15597 and *E. coli* 700891 (PFU · 100 ml^{-1}), enteric viruses (MPN · 100 $liters^{-1}$), *Giardia* total counts (cysts · 100 $liters^{-1}$), and *Cryptosporidium* total and viable counts (oocysts · 100 $liters^{-1}$) are shown. Detection limits were used as concentrations for parameters that were nondetectable. The boxes represent 50% of the data, the vertical lines represent the mean, the lines extending from the boxes represent the 95% confidence limits, and the individual data points represent outliers.

Statistical analysis. Statistical analyses were conducted using SAS software version 8.2 (SAS Institute, Cary, NC) or SPSS version 12.0. Data distributions were evaluated with the Shapiro-Wilk test, which was conducted on the raw data, log_{10}-transformed data, and square-root-transformed data. Nonparametric statistical tests were utilized for nonnormally distributed data. Parametric tests were used for analysis of variance, and the Tukey post-hoc test was used to compare treatment means. The Spearman rank correlation was used to test the relationship between indicator organism and pathogen concentrations in the final effluent. A binary logistic regression model (SPSS 12.0) was utilized to determine whether indicator organism concentrations predicted the probability of the occurrence of pathogens in disinfected effluent samples. The dependent variable (pathogen) was treated as a binary variable; that is, a score of 0 was assigned when the organism was not detected, and a score of 1 was assigned when the organism was detected. The independent variables were continuous, and values for samples in which organisms were not detected were reported as 0. True-positive, true-negative, false-positive, and false-negative values were calculated as the number of samples falling into each category divided by the total sample number.

Discriminant analysis was performed on data from effluent samples by using the DISCRIM procedure of SAS (prior probabilities, equal; covariance matrix, pooled). The results of six assays for indicator organisms (total coliform, fecal coliform, *C. perfringens*, enterococci, and F-specific coliphage assays on two hosts) were converted into a string of binary variables representing the presence or absence of each indicator. The ability of the indicator data string to predict the presence or absence of each pathogen (*Giardia*, *Cryptosporidium*, and enteric viruses) was assessed separately. Results are expressed as the percentage of samples correctly classified into the "pathogen present" and "pathogen absent" categories.

RESULTS

The results presented here represent multiple samplings from six facilities producing reclaimed water and focus on microbial concentrations in the influent and in the reclaimed water (disinfected effluent), which is distributed to end users.

Microbial concentrations through treatment. Concentrations of indicator organisms and pathogens before (untreated wastewater) and after (disinfected effluent) treatment are shown in Fig. 1 in a box plot format. The limit of detection (see Materials and Methods) was substituted for measured values for samples in which the organism was not detected, which was rare in influent samples but common in effluent samples. Total coliform concentrations were the highest of the microbial measurements in influent samples ($>10^7$ CFU · 100 ml^{-1}), followed by fecal coliforms and enterococci ($\sim10^6$ CFU · 100 ml^{-1}) (Fig. 1). *Clostridium perfringens* values ranged from 10^4 to $>10^6$ CFU · 100 ml^{-1}. Coliphage levels were highly variable, ranging from 10^3 to 10^8 PFU · 100 ml^{-1}. Pathogen concentrations in the influent (Fig. 1) were 4 to 5 orders of magnitude lower than indicator organism concentrations (note that the unit for pathogen concentrations is 100 $liters^{-1}$). It should be noted that while the enteric virus concentrations represent infectious viruses, *Cryptosporidium* and *Giardia* concentrations reflect the total number of cysts or oocysts (infectious and noninfectious) viewed under immunofluorescence microscopy. In the influent samples, about 40% of the detected *Cryptosporidium* organisms were infective as defined by the focus detection method-MPN cell culture assay. Microbial concentrations in disinfected effluents were much lower, as expected (Fig. 1), and in most cases were near or below the detection limits for each assay.

The percentages of samples from each treatment step that contained detectable levels of each indicator organism and pathogen are summarized in Table 2. Total and fecal coliforms, enterococci, and coliphages were detected in 100% of

TABLE 2. Percentage of samples with detectable indicator organisms and pathogens

Indicator or pathogen	% of samples positive in each stage[a]			
	Influent	Biological treatment	Filter effluent	Disinfected effluent
Indicators				
Total coliforms	100	100	94	63
Fecal coliforms	100	97	65	27
Enterococci	100	94	84	27
Clostridium perfringens	93	86	79	61
Coliphages on 15597	100	97	83	38
Coliphages on 700891	100	93	80	45
Pathogens				
Enteric viruses	100	73	58	31
Giardia	100	94	88	80
Cryptosporidium				
Total oocysts	74	84	71	70
Infectious oocysts	32	19	19	20

[a] Data from all sampling events at the six facilities were pooled for each treatment step.

influent (untreated) wastewater samples, in which detection limits were generally 33.3 CFU or PFU · 100 ml^{-1}. Three of the 30 untreated wastewater samples were below the detection limit for *C. perfringens* (33.3 CFU · 100 ml^{-1}). Enteric viruses (detection limit, 100 MPN · 100 $liters^{-1}$) and *Giardia* (detection limit, 500 cysts · 100 $liters^{-1}$) were also found in 100% of untreated wastewater samples. *Cryptosporidium* oocysts were detected in 74% of the untreated wastewater samples; however, infective oocysts were identified in only 32% of these samples. The detection limit for *Cryptosporidium* in the influent samples depended upon the volume that could be filtered and ranged from 300 to 2,100 oocysts · 100 $liters^{-1}$. Following biological treatment, the concentrations of indicators and pathogens were reduced by about 1 to 2 $\log_{10}$, thus decreasing the frequency of detection of most organisms; i.e., enteric viruses were detected in only 73% of the secondary effluent samples, compared to 100% of the influent samples. The frequency of detection of *Cryptosporidium* increased from 75% in the influent samples to 84% in the secondary effluent samples, due to the more sensitive detection limits in secondary effluent (21 to 94 oocysts · 100 $liters^{-1}$); however, the frequency of detection of infectious oocysts decreased from 32% to 19%. Filtration further decreased the frequency of detection of microorganisms, particularly for enterococci, the coliphages, and *Giardia* (Table 2).

In disinfected samples, total coliforms and *C. perfringens* were detected most frequently, and fecal coliforms and enterococci were least frequently detected (Table 2). While the frequencies of detection of fecal coliforms and enterococci in disinfected effluents were similar (27%), they were simultaneously detected in only one sample, whereas either fecal coliforms or enterococci were detected in 50% of the samples. An assessment of the correlation between total residual chlorine and fecal coliform concentrations in treated effluent samples from all the facilities showed no significant relationship between these parameters (data not shown).

Pathogens, measured on the scale of 100 $liters^{-1}$, were detected in 80% (*Giardia*) to 31% (enteric virus) of samples. Both *Giardia* and *Cryptosporidium* were detected by microscopy in 60% of disinfected effluent samples. Unlike the trend noted for the other organisms, the percentage of samples in which *Cryptosporidium* oocysts were detected remained fairly consistent through the treatment stages (71 to 84%); however, detection limits became progressively more sensitive through the treatment stages, reaching 2.2 to 6.9 oocysts · 100 $liters^{-1}$ in the reclaimed water (disinfected effluents). The percentage of samples containing detectable levels of infectious oocysts decreased from 32% in the untreated wastewater samples to 20% in the reclaimed water samples.

The frequency of detection of the various microorganisms in disinfected effluent samples was compared using Fisher's exact test. Total coliforms and *C. perfringens* were detected in significantly more samples (63% and 61%, respectively) than enterococci or fecal coliforms (both 27%). Other proportional comparisons between indicator organism detections were not significantly different. The protozoan parasites were detected in significantly more disinfected effluent samples than enteric viruses, but there was no significant difference in the proportion of samples in which *Giardia* cysts versus *Cryptosporidium* oocysts were detected. Infective *Cryptosporidium* was detected in significantly fewer disinfected effluent samples than total *Giardia* or *Cryptosporidium*.

Of all the indicator organisms, including the coliphages, the fecal coliforms were found at the lowest concentrations in final effluent samples (Fig. 1) and were among the least frequently detected (Table 2). At hypothetical detection limits of 2 CFU · 100 ml^{-1}, total coliforms would be detected in 43% of the disinfected effluent samples, whereas fecal coliforms would be detected in only 10% of the samples ($n = 30$). Reducing the detection limit to 0.2 CFU · 100 ml^{-1} (the actual detection limit) increased the frequency of detection of fecal coliforms and total coliforms to 27% and 63%, respectively. The relationship between hypothetical detection limit and detection frequency was log linear ($r^2 = 0.96$ for total coliforms and 0.94 for fecal coliforms).

Predictive relationships between microorganisms. Data from disinfected effluent samples were analyzed separately (by facility) and as a pooled data set (all facilities) to determine if the concentrations of any of the indicators (total coliforms, fecal coliforms, enterococci, *C. perfringens*, or coliphages) were correlated with each other or with concentrations of pathogens (enteric viruses, *Giardia*, or *Cryptosporidium*). Analysis of results by facility did not yield significant correlations (probably due to small sample sizes); however, significant correlations between indicator organism concentrations were observed in the pooled data sets: i.e., for total coliform and fecal coliform (Spearman's $r_s = 0.5986$; $P = 0.0005$), *C. perfringens* versus coliphages on host *E. coli* 15597 ($r_s = 0.5303$; $P = 0.0031$), *C. perfringens* versus coliphages on host *E. coli* 700891 ($r_s = 0.4981$; $P = 0.0060$), and coliphages on the two *E. coli* hosts ($r_s = 0.7915$; $P < 0.0001$). No significant correlation between concentrations of any combination of indicator organism and pathogen was observed.

Enteric viruses were above detection limits in 31% of the disinfected effluent samples ($n = 30$); however, coliphages and enteric viruses co-occurred in only 13% of the disinfected effluent samples. Concentrations of coliphages on both *E. coli* hosts were plotted against enterovirus concentrations using

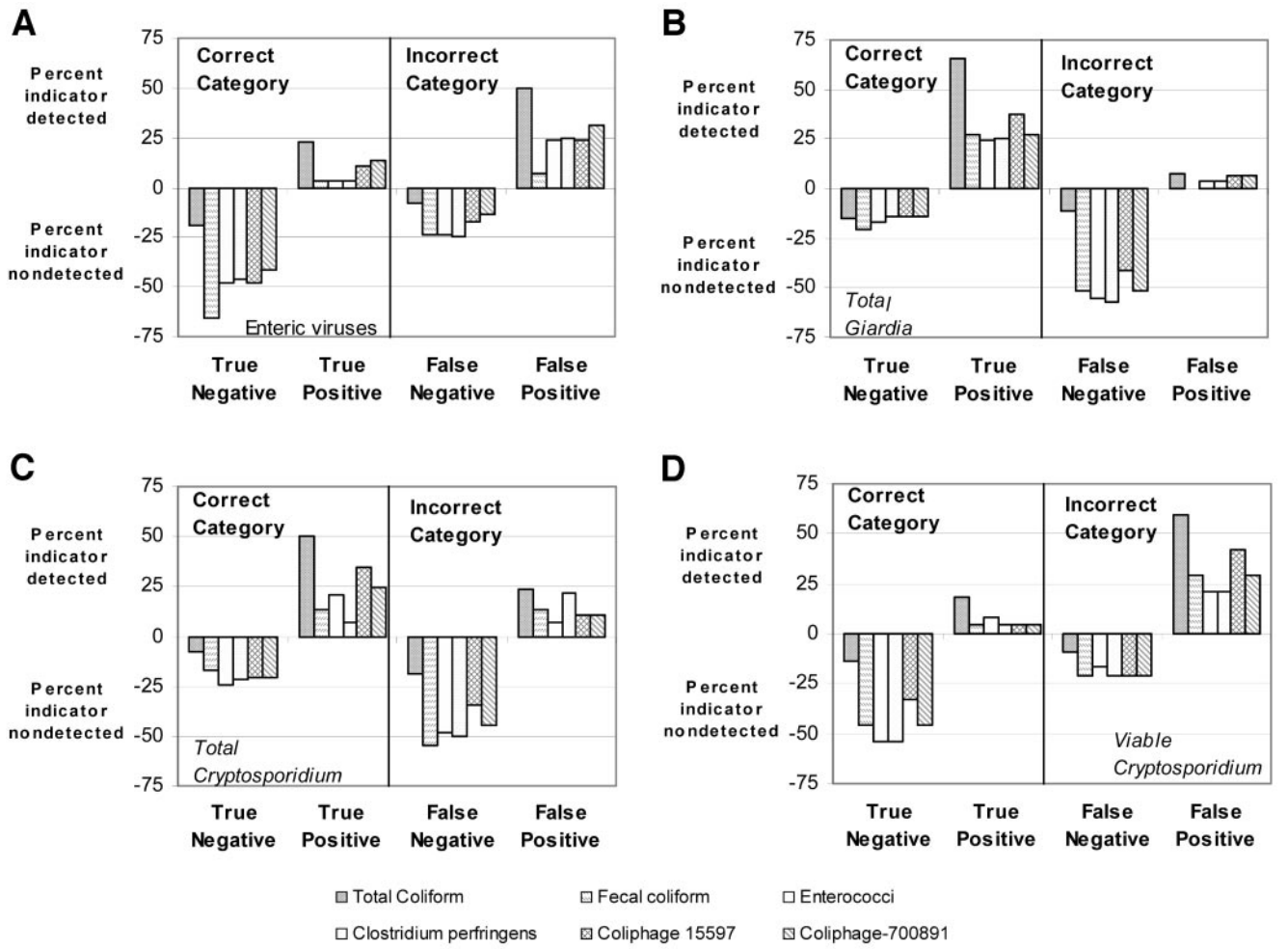

FIG. 2. Relationship between detection of individual indicators and accuracy of pathogen detection in disinfected effluent. All percentages were calculated from the total sample number. Detection limits were 0.2 CFU · 100 ml^{-1} for total and fecal coliforms, enterococci, and *Clostridium perfringens* and 10 PFU · 100 ml^{-1}. for coliphages. (A) Enteric viruses; (B) *Giardia* cysts; (C) *Cryptosporidium* oocysts; (D) infectious *Cryptosporidium*.

only samples in which coliphages and enteric viruses were detected, but the slopes of the relationships were not significantly different from 0 (data not shown).

Binary logistic regression was used to test the hypothesis that indicator organism concentrations were predictive of the presence or absence of pathogens in disinfected effluent. Observations of enteric viruses, *Cryptosporidium* oocysts, and *Giardia* cysts were converted to binary data, and the relationship between the concentration of each indicator organism and the presence or absence of each pathogen was assessed, as well as the relationships between the pathogens. Nagelkerke's *R*-square, which can range from 0.0 to 1.0, denotes the strength of the association; stronger associations have values closer to 1.0. Three indicator-pathogen combinations displayed very weak correlations: coliphage concentration (host, *E. coli* 15597) and enteric virus presence/absence (*R*-square = 0.226), fecal coliform concentrations and *Giardia* presence/absence (*R*-square = 0.222), and total coliforms and infectious *Cryptosporidium* presence/absence (*R*-square = 0.241). In each case, the variability in x accounted for only a fraction of the variability in y (odds that a pathogen would be present). A much tighter association was evidenced, for example, between the two coliphage assays on different hosts (*R*-square = 0.762), as would be expected for the two similar assays. No correlations between indicators and pathogens were found using the Spearman correlation; however, this is not unusual as binary logistic regression relies on maximum likelihood, does not require linear relationships between variables (19), and utilizes a binary (0, 1) dependent variable.

The analytical consequences of the failure of indicators to correlate with pathogens are shown in Fig. 2. True negatives are samples in which neither indicators nor pathogens were detected, true positives are samples in which both indicators and pathogens were detected, false negatives are samples in which pathogens were detected when indicators were not detected, and false positives are samples in which indicators were detected when pathogens were not detected. These values add up to 100% for each indicator-pathogen combination. Total coliforms frequently survived the disinfection process; therefore, they tended to be present when pathogens were present,

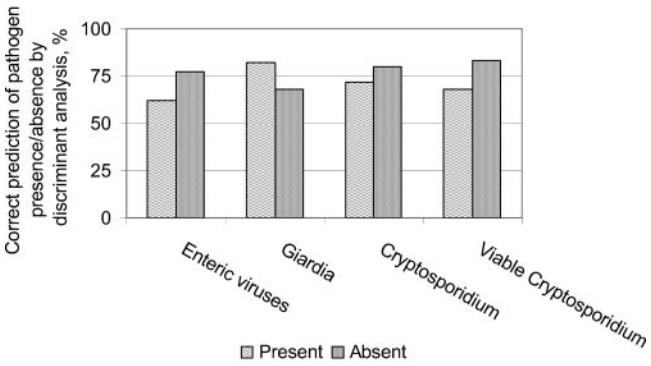

FIG. 3. Discriminant analysis, with results showing the percentage of samples correctly categorized with respect to presence or absence of each pathogen. All of the indicators were used as binary dependent variables. Present, percentage of samples with pathogens actually present and in which pathogen presence was predicted by DA. Absent, percentage of samples in which pathogens were not detected and in which pathogen absence was predicted by DA.

resulting in a relatively high true-positive rate compared to the other indicators (Fig. 2A to D). However, total coliforms also tended to have a low true-negative rate (which would ideally be high) and a relatively high false-positive rate, particularly in the cases of enteric viruses and viable *Cryptosporidium*. In contrast, fecal coliforms, which were relatively infrequently detected in disinfected effluent, tended to have a high true-negative rate but also a low true-positive rate. The percentage of results in the correct categories (true positive and true negative) was not much greater than 50% for any of the indicator-pathogen combinations, although ideally these categories would comprise 100% of observations. Each type of correct and incorrect categorization has distinct implications for public health protection (see Discussion).

DA is a multivariate statistical technique that can be used to classify observations into categories based on a series of independent variables. DA was used to test the hypothesis that the presence or absence of indicator organisms in disinfected effluent samples could predict the presence versus absence of each pathogen (Fig. 3). Indicator organism data for each sample were represented as a string of six binary variables (presence or absence of total coliforms, fecal coliforms, enterococci, *C. perfringens*, coliphages on *E. coli* 15597, and coliphages on *E. coli* 700891). The presence or absence of each of the pathogens was relatively accurately predicted by the suite of indicator organism data for the 29 effluent samples analyzed (Fig. 3). The data are presented as (i) the percentage of samples with pathogens actually present in which pathogen presence was predicted by DA and (ii) the percentage of samples in which pathogens were actually not detected and in which pathogen absence was predicted by DA. When pathogen-positive and pathogen-negative samples were considered together, 72% of enteric virus samples, 79% of *Giardia* samples, 75% of *Cryptosporidium* oocyst samples, and 71% of infectious *Cryptosporidium* samples were placed in the correct category (presence or absence of the pathogen) by discriminant analysis. The absence of all pathogens except *Giardia* was more accurately predicted than pathogen presence. In most cases, removal of one variable (indicator organism) from the data string caused the correct classification rate to decrease by a few percentage points, as one or two additional observations would be misclassified. No single indicator was most highly predictive of membership in the "presence" or "absence" category for pathogens. Interestingly, when coliphage assayed on *E. coli* 700891 was excluded as a variable, it improved the results of the enteric virus analysis by correctly categorizing one additional "presence" sample.

DISCUSSION

The current monitoring approach to assess the microbial safety of reclaimed water is the measurement of total or fecal coliform concentrations in a single daily grab sample. Utilities and regulatory agencies have assumed a predictive relationship between indicator organism and pathogen levels to protect the public from exposure to pathogens; however, the imperfect relationship between coliform bacteria and pathogens, such as viruses (12, 13, 25) and protozoa (5), through wastewater treatment has been known for some time (see reference 16 for a review). A major goal of this work was to examine monitoring strategies and to determine whether any predictive relationship between conventional and alternative indicator organisms and pathogens in reclaimed water could be discerned among a group of treatment facilities producing reclaimed water.

Detection of microorganisms. Log_{10} reduction of microorganisms through wastewater treatment trains is frequently reported (23, 24) but should not be relied upon as the sole measurement of treatment efficacy. Organisms with very high initial concentrations may experience large log reductions while maintaining detectable levels in disinfected effluents, as illustrated by the total coliforms in this study. Total coliforms experienced an average $\log_{10}$ reduction of >7 from influent to final effluent but were still detected in 67% of disinfected effluent samples.

The linear relationship between hypothetical detection limits and the percentage of samples in which total or fecal coliforms would be detected demonstrates the usefulness of larger sample volumes for detecting indicators, but this ability did not generally translate to a significant predictive relationship between indicators and pathogens. However, if normal volumes (100 ml) had been assayed for fecal coliforms and if we assume that no detection would have occurred in samples in which <1 CFU/100 ml was present, the weak correlations between fecal coliforms versus *Giardia* presence or absence and total coliforms versus infectious *Cryptosporidium* presence or absence would not have been detected (data not shown).

Bacteriophages have been suggested as an alternative indicator for enteric viruses, as their morphology and survival characteristics resemble those of some of the enteric viruses (13, 29). This study found a weak but significant relationship between the presence or absence of enteroviruses and coliphages on *E. coli* 15597 by binary logistic regression. A significant relationship was not found between enteroviruses and coliphages on *E. coli* 700891. This observation, coupled with the improvement in prediction of enterovirus presence or absence by discriminant analysis when coliphage on *E. coli* 700891 was removed as a variable, suggests that the use of other *E. coli* hosts for coliphage assays should be further explored.

The use of U.S. EPA method 1623 for detection of *Crypto-*

sporidium oocysts does not permit determination of oocyst viability or infectivity, which is crucial information for assessment of the human health risk associated with this parasite. The focus detection method of detecting infectious *Cryptosporidium* (27) has been utilized in a number of studies (11, 15, 21, 22, 26–28, 34), and results coincide well with those of mouse infectivity assays (15). Approximately one-quarter of the disinfected effluent samples with detected *Cryptosporidium* oocysts had detectable levels of infectious *Cryptosporidium*, a disturbing observation in that reclaimed water represents a potential human exposure pathway, depending on how the reclaimed water is used. None of the indicators correlated with *Cryptosporidium* oocysts or infectious *Cryptosporidium*.

Because indicators were not predictive of pathogen presence, the results yielded a high percentage of false-negative or false-positive results for all indicator-pathogen combinations. The relationship of indicators with pathogens that were detected more frequently, such as *Giardia*, tended to show a greater frequency of false negatives (indicators absent but pathogens present). The relationship of indicators with pathogens that were less frequently detected, such as enteric viruses and infectious *Cryptosporidium*, generally showed a higher frequency of false-positives (indicators present but pathogens absent). False-positive results are undesirable because they represent "false alarms." An indicator that is frequently present in the absence of pathogens, such as total coliforms in this study, is not very informative as to the true risk to human health but is relatively conservative in terms of human health protection. False negatives, on the other hand, suggest that probable human health risks are not being detected, which certainly compromises efforts to protect public health. This study suggests that choosing one indicator to predict the survival and/or occurrence of a wide variety of microbial pathogens forces a choice between the two types of error.

Although individual indicator organisms and pathogens were weakly correlated or uncorrelated, the use of discriminant analysis on the composite data set resulted in the relatively accurate prediction of the presence or absence of enteric viruses, *Giardia*, *Cryptosporidium* oocysts, and infectious *Cryptosporidium*. With the exception of *Giardia*, errors tended to be false negatives, as the absence of enteric viruses and *Cryptosporidium* was more accurately predicted than their presence. Further analysis of larger data sets and other indicators, perhaps coupled with measurement of key pathogens, may allow us to refine the predictive capabilities demonstrated by this multivariate analysis. Such a monitoring strategy should protect public health better than the one-indicator system currently used.

ACKNOWLEDGMENTS

We acknowledge the technical help of Molly R. McLaughlin, Stefica Depovic, Angela Gennaccaro, and Tracie Jenkins.

Funding was provided by the Water Environment Research Foundation, project 00-PUM-2T.

REFERENCES

1. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers, New York, N.Y.
2. **American Public Health Association.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Inc., Washington D.C.
3. **Anonymous.** 1998. Reuse of reclaimed water and land application, State of Florida, 62-610. www.dep.state.fl.us/legal/rules/wastewater/62-610.pdf.
4. **Bisson, J., and V. Cabelli.** 1979. Membrane filtration enumeration method for *Clostridium perfringens*. Appl. Environ. Microbiol. **37:**55.
5. **Bonadonna, L., R. Briancesco, M. Ottaviani, and E. Veschetti.** 2002. Occurrence of Cryptosporidium oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environ. Monit. Assess. **75:**241–252.
6. **California Administrative Code.** 1978. Wastewater reclamation criteria. California Admistrative Code, title 22, division 4. California Department of Health Services, Berkeley.
7. **Cosgrove, W. J., and F. R. Rijsbermann.** 2000. World water vision-making water everybody's business. Earthscan Publications, London, United Kingdom.
8. **Debartolomeis, J., and V. J. Cabelli.** 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. Appl. Environ. Microbiol. **57:**1301–1305.
9. **Fujioka, R. S., and L. K. Shizumura.** 1985. Clostridium perfringens, a reliable indicator of stream water quality. J. Water Pollut. Control Fed. **57:**986–992.
10. **Gantzer, C., A. Maul, J. M. Audic, and L. Schwartzbrod.** 1998. Detection of infectious enteroviruses, enterovirus genomes, somatic coliphages, and *Bacteroides fragilis* phages in treated wastewater. Appl. Environ. Microbiol. **64:** 4307–4312.
11. **Gennaccaro, A. L., M. R. McLaughlin, W. Quintero-Betancourt, D. E. Huffman, and J. B. Rose.** 2003. Infectious *Cryptosporidium parvum* oocysts in final reclaimed effluent. Appl. Environ. Microbiol. **69:**4983–4984.
12. **Goyal, S. M., W. N. Adams, M. L. O'Malley, and D. W. Lear.** 1984. Human pathogenic viruses at sewage sludge disposal sites in the Middle Atlantic region. Appl. Environ. Microbiol. **48:**758–763.
13. **Havelaar, A. H., M. van Olphen, and Y. C. Drost.** 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. Appl. Environ. Microbiol. **59:**2956–2962.
14. **Hsu, F.-C., A. Chung, A. Amante, Y.-S. C. Shieh, D. Wait, and M. D. Sobsey.** 1996. Presented at the AWWA Water Quality Technology Conference.
15. **Joachim, A., E. Eckert, F. Petry, R. Bialek, and A. Daugschies.** 2003. Comparison of viability assays for Cryptosporidium parvum oocysts after disinfection. Vet. Parasitol. **111:**47–57.
16. **Leclerc, H., D. A. Mossel, S. C. Edberg, and C. B. Struijk.** 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annu. Rev. Microbiol. **55:**201–234.
17. **Levine, A. D., and T. Asano.** 2004. Recovering sustainable water from wastewater. Environ. Sci. Technol. **30:**201A–208A.
18. **Miescier, J. J., and V. J. Cabelli.** 1982. Enterococci and other microbial indicators in municipal wastewater effluents. J. Water Pollut. Control Fed. **54:**1599–1606.
19. **Motulsky, H.** 1995. Intuitive biostatistics. Oxford University Press, New York, N.Y.
20. **Payment, P., and E. Franco.** 1993. *Clostridium perfringens* and somatic coliphages as indicators of the efficiency of drinking water treatment for viruses and protozoan cysts. Appl. Environ. Microbiol. **59:**2418–2424.
21. **Pokorny, N. J., S. C. Weir, R. A. Carreno, J. T. Trevors, and H. Lee.** 2002. Influence of temperature on Cryptosporidium parvum oocyst infectivity in river water samples as detected by tissue culture assay. J. Parasitol. **88:**641–643.
22. **Quintero-Betancourt, W., A. L. Gennaccaro, T. M. Scott, and J. B. Rose.** 2003. Assessment of methods for detection of infectious *Cryptosporidium* oocysts and *Giardia* cysts in reclaimed effluents. Appl. Environ. Microbiol. **69:**5380–5388.
23. **Rose, J. B., L. J. Dickson, S. R. Farrah, and R. P. Carnahan.** 1996. Removal of pathogenic and indicator microorganisms by a full-scale water reclamation facility. Water Res. **30:**2785–2797.
24. **Rose, J. B., D. E. Huffman, K. Riley, S. R. Farrah, J. O. Lukasik, and C. L. Hamann.** 2001. Reduction of enteric microorganisms at the Upper Occoquan Sewage Authority water reclamation plant. Water Environ. Res. **73:**711–720.
25. **Simpson, D., J. Jacangelo, P. Loughran, and C. McIlroy.** 2003. Investigation of potential surrogate organisms and public health risk in UV irradiated secondary effluent. Water Sci. Technol. **47:**37–43.
26. **Slifko, T. R., D. Friedman, J. B. Rose, and W. Jakubowski.** 1997. An in vitro method for detecting infectious *Cryptosporidium* oocysts with cell culture. Appl. Environ. Microbiol. **63:**3669–3675.
27. **Slifko, T. R., D. E. Huffman, and J. B. Rose.** 1999. A most-probable-number assay for enumeration of infectious *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. **65:**3936–3941.
28. **Slifko, T. R., E. Raghubeer, and J. B. Rose.** 2000. Effect of high hydrostatic pressure on Cryptosporidium parvum infectivity. J. Food Prot. **63:**1262–1267.
29. **Turner, S. J., and G. D. Lewis.** 1995. Comparison of F-specific bacteriophage, enterococci, and faecal coliform densities through a wastewater treatment process employing oxidation ponds. Water Sci. Technol. **31:**85–89.
30. **U.S. Environmental Protection Agency.** 1996. ICR microbial laboratory manual. EPA/600/R-95/178. U.S. Environmental Protection Agency, Washington, D.C.
31. **U.S. Environmental Protection Agency.** 2000. Improved enumeration methods for the recreational water quality indicators: enterococci and *Escherichia coli*. EPA-821/R-97/004. U.S. Environmental Protection Agency, Washington, D.C.

32. **U.S. Environmental Protection Agency.** 1997. Method 1600: membrane filter test methods for enterococci in water. EPA-821/R-97/004. U.S. Environmental Protection Agency, Washington, D.C.
33. **U.S. Environmental Protection Agency.** 2001. Method 1623: *Cryptosporidium* and *Giardia* in water by filtration/IMS/FA. EPA-821-R-01-025. U.S. Environmental Protection Agency, Washington, D.C.
34. **Weir, S. C., N. J. Pokorny, R. A. Carreno, J. T. Trevors, and H. Lee.** 2001. Improving the rate of infectivity of Cryptosporidium parvum oocysts in cell culture using centrifugation. J. Parasitol. **87:**1502–1504.
35. **World Health Organization.** 1989. Health guidelines for the use of wastewater in agriculture and aquaculture. World Health Organization, Geneva, Switzerland.