Evaluation of three source tracking indicator organisms for watershed management

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ABSTRACT

The use of indicator organisms to ensure the microbial safety of drinking water is a standard practice throughout the developed world. Traditional coliform indicators, however, are not always the most suitable indicator for all waterborne pathogens, especially viruses and protozoans. Drinking water contaminated with human waste is typically associated with a higher risk of infection by human pathogens. For that reason, source-specific or source tracking indicators would be beneficial in identifying the source of water contamination. The focus of this research was to determine the potential of three source-specific indicators (sorbitol-fermenting *Bifidobacteria*, *Rhodococcus coprophilus* and serogroups of F-specific coliphages) for differentiating human, grazing animal and other wildlife microbial inputs into a drinking water watershed. Three locations within a surface water source watershed management area were sampled over a 13-month period. The results indicated that the three indicators tested might be a good means of discriminating between microbial input sources into drinking water supplies.

Key words | *Bifidobacteria*, coliphage, F-specific coliphage, *Rhodococcus coprophilus*

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INTRODUCTION

One goal of watershed management is to provide the highest possible quality raw water to a drinking water system. Water suppliers are having an increasingly difficult time attaining this goal because of growing populations and subsequent increased land development that exposes drinking water sources to a number of potential pollutant sources. Outbreaks of waterborne diseases are being detected more frequently in cases where total and fecal coliform standards have been met (Fox & Lytle 1996; DeNileon 1998).

It is important for source water managers to be able to distinguish between microbial input sources in order to decide upon appropriate remedial actions. Tools used to discriminate between microbial inputs from humans, agricultural animals and wildlife are necessary. These tools should be cost-effective and easily applied using the resources and expertise currently available. To be widely adopted, new microbial indicators should be enumerated using techniques that water utility laboratory personnel can learn quickly and apply easily. An ideal indicator must be present when pathogens are present and absent when pathogens are absent as well as being equally or more persistent in the environment as the pathogen for which it is indicating (Gerba 1987; Sobsey *et al.* 1995). These principles apply to source-specific indicators, although the surrogate's survival rates do not necessarily need to match those of pathogens if the surrogate is to be used for the purpose of source identification. Three potential alternative indicators are sorbitol-fermenting *Bifidobacteria*, *Rhodococcus coprophilus* and serogroups of F-specific coliphages.

Sorbitol-fermenting *Bifidobacteria* have been associated with human sewage in several studies (Mara & Oragui 1983; Jagals *et al.* 1995; Long *et al.* 2002). For example, the organism was enumerated from all human related samples tested, consisting of 22 human fecal samples, 11 raw sewage and 12 treated effluent samples (Mara & Oragui 1983). In the same study, the indicator

was absent from all cattle (7), sheep (7), pig (11), horse (9), rabbit (7), rat (7), mice (3), hen/cock (8), cat (4) and dog (4) fecal samples using HBSA medium. All of these animals were domesticated. In addition, Mara & Oragui (1983) tested ten wild mice and rat fecal samples; all were negative for sorbitol-fermenting *Bifidobacteria*. The reported results constituted the entire range of animal feces tested, all being negative for this indicator.

The indicator *Rhodococcus coprophilus* has often been isolated in samples collected from agricultural animals (Rowbotham & Cross 1977; Mara & Oragui 1985*a*; Savill *et al.* 2001; Long *et al.* 2002). The indicator was reported to be absent from 34 human samples, while present in all cattle (8), sheep (7), pig (8), horse (5), duck (5) and goose (3) samples tested (Mara & Oragui 1985*a*). These animals were agricultural animals, rather than wild animals. The indicator was also absent from all cat (5), rabbit (4), rat (4), mouse (28 composite) and turkey (6) fecal samples. Samples from dog, hens from various sources, and seagulls were occasionally positive for *R. coprophilus* (exact numbers not reported). In another study, six cow, three sheep, one horse and one deer sample were demonstrated by both plate count and PCR methods to contain *R. coprophilus* (Savill *et al.* 2001). In the same study, five human, one pig, one possum, one rabbit and one duck sample were all negative by both plate count and PCR methods. Neither indicator was detected in the feces of wildlife in a study conducted in the United States (Long *et al.* 2002). However, *R. coprophilus* was detected in a number of wild animal fecal samples in a study conducted in Nigeria and Zimbabwe, although all were grazing animal species such as elephants, giraffes and zebra (Mara & Oragui 1985*b*).

The enumeration of these indicator organisms directly from water samples is hypothesized to be indicative of microbes from their related sources reaching a water body. Sorbitol-fermenting *Bifidobacteria* appear to be indicators of recent (in time and space) human fecal contamination owing to its relatively short environmental survival in surface waters as a strict anaerobe (Rhodes & Kator 1999; Long *et al.* 2002). *R. coprophilus* can survive for considerable amounts of time in environmental waters, at least 2 weeks, making it an indicator of recent or distant (in time and space) contamination (Arango 2000).

F + RNA coliphages are a type of virus that infects male-specific strains of *E. coli* bacteria and are found in the intestines of various warm-blooded animals. Chung (1993) summarized a body of research investigating the occurrence of F-specific coliphages in individual fecal samples. Of 652 human fecal samples, 2% were positive for F-specific coliphages. For animal fecal samples, the percentages of F-specific coliphage positive samples were 26% of 66 pig feces, 5% of 64 cow samples, 3% of 30 horse samples, 100% of 28 chicken manure samples, and none of the other animal fecal samples tested including mouse, rabbit and various fowl species. Overall, the percentage of individuals that carry F-specific coliphages is low; however, those carrying the viruses shed them in large numbers (Chung 1993; El-Khoury 2003).

The results from research have demonstrated that serotyping of F + RNA coliphages can distinguish between inputs from human and warm-blooded animal/non-human sources (Havelaar *et al.* 1986; Havelaar 1987; Gerba 1987; Chung 1993; Sobsey *et al.* 1995; Schaper *et al.* 2002). These serogroups are:

Group I non-human animals;
Group II primarily human feces and occasionally pig feces;
Group III exclusively human; and
Group IV primarily non-human origin with rare human associations (Gerba 1987).

F-specific coliphages are enumerated from samples and individual isolates subject to serotyping in order to discriminate between human and non-human microbial sources. Again, Chung (1993) examined a body of F-specific coliphage serotyping research. With the exception of one pig isolate of 267 animal isolates tested, no Group II or III human related isolates were identified. The animal isolates were from pigs, cattle, calves, sheep, horses, zoo animals and chickens. El-Khoury (2003) also found no Group II or III isolates among the 35 F + RNA coliphages isolated from 22 avian guano samples from around the United States. Of 1,079 domestic wastewater isolates tested, 710 or 66% were Group II or III coliphages, and the remaining 34% were Group I or IV, animal related (Chung 1993). However, these numbers are skewed by a single study in which 272 of 300 isolates from

Japanese treatment plants were either Group I or IV. Excluding these numbers, 88% of the isolates tested from domestic wastewater were of the human related serotypes. Similar data for individual human feces is not available.

The goal of this research was to determine the potential for the three described alternative source-specific indicators for differentiating between human, grazing animal and non-human fecal contamination in environmental waters. In addition, the study focused on bacteriologically based methods, the rationale being that these methods could be more easily adopted by water utility laboratories without the addition of expensive instrumentation or extensive training for personnel. To determine their usefulness as indicator organisms, a year-long study of three sites with defined associated land uses was conducted. The sites represented a highly protected area, a forested buffer zone and a residential/commercial developed area in the Wachusett Reservoir watershed in Central Massachusetts, managed by the Metropolitan District Commission (MDC). The three source-specific organisms as well as several traditional microbial and chemical water quality characteristics were measured for each sample.

METHODOLOGY

The Wachusett Reservoir watershed, located in Central Massachusetts, is an example of a water source subject to potential environmental microbial inputs from septic systems, domestic animals, wildlife and agricultural runoff (Shafer 2001). Three sampling sites were chosen based on their surrounding land uses and existing MDC water quality monitoring data. Site 1 is located in Clinton, Massachusetts, and is where water enters the distribution system. Site 1 was chosen to represent highly protected sites and was hypothesized to contain low to non-existent levels of indicator organisms. Sites 2 and 3 are both tributaries to the Wachusett Reservoir and are located in Boylston, Massachusetts. Approximately 1.6 km (one mile) upstream of Site 2 is a residential area and a small-scale dairy operation, but the major impact on water quality is derived from wildlife such as deer and ducks. Site 3 is located within a highly developed residential and commercial area. The major impact on water quality is runoff from roads and filtrate from septic system leach fields. Site 3 is also subject to runoff from two small horse stables located within about 3 km (two miles) upstream of the sampling site.

Samples were collected monthly from March 1998 to March 1999. Samples were collected in sterile, one-litre, high density polyethylene bottles which were placed in coolers at approximately 2–4°C containing blue-paks, then transported to University of Massachusetts (UMass) laboratories. All samples were analysed within 6 h of collection. Site 1 samples were collected from a tap that runs continuously at a Massachusetts Water Resources Authority (MWRA) utility building. Site 2 and 3 samples were collected in the tributaries by submerging the bottles while keeping the sampler's hand on the downstream side and moving the bottle upstream as the sample was collected. This was to minimize both contamination from the sampler's hands and disturbed sediment.

It is recognized that the indicators in question can be analysed using more highly sophisticated molecular-based methods (Hsu *et al.* 1995; Savill *et al.* 2001; Lynch *et al.* 2002). However, one focus of this project was to utilize methods that could be transferred widely to water utility laboratories without the addition of expensive instrumentation or specialized personnel. If the use of less exacting bacteriologically based field monitoring of these source-specific indicators proves useful, a water management authority could choose to apply molecular-based methods as desired.

Sorbitol-fermenting *Bifidobacteria* were enumerated using a membrane filtration presumptive step followed by a biochemical reaction confirmation procedure adapted from Beerens (1991) and Mara & Oragui (1985*a*) for use in UMass laboratories (Arango 2000; Long *et al.* 2002). The filtration apparatus and membranes used are identical to those used for membrane filtration analysis of coliforms. Briefly, appropriate volumes (1–500 ml) of sample water were aseptically measured and filtered through prewetted 0.45 μm (Type HA) gridded membranes (MSI brand, Fisher Scientific, Fair Lawn, New Jersey). The filter tower was rinsed with sterile phosphate buffered saline (PBS) and vacuum aspirated. The membranes were then transferred onto human bifid sorbitol agar (HBSA). The plates

were allowed to remain exposed to ambient air conditions for only up to 2 h. Inoculated plates were inverted and incubated under anaerobic conditions (BBL GasPak Jar Systems; Fisher Scientific, Fair Lawn, New Jersey) at 35–37°C for 46–50 h. After incubation, the plates were counted for yellow or mustard coloured, domed and mucoid colonies that were considered sorbitol-fermenting *Bifidobacteria* presumptive positives.

A representative number of presumptive positive colonies were transferred within 2 h onto reinforced clostridial agar (RCA, Difco, Detroit, Michigan) for confirmation testing. Organisms which only grew on the anaerobic RCA plate (strict anaerobes) and presented a single colony morphology were then Gram stained, catalase tested and inoculated into glucose, lactose and sorbitol purple broth fermentation tubes, motility agar and nitrate broth tubes. Organisms that were Gram variable, bifurcating rods that were non-motile, catalase and nitrate reduction negative and that fermented glucose, lactose and sorbitol, and produced acid but not gas were confirmed as sorbitol-fermenting *Bifidobacteria*. Colony morphologies and confirmation reactions were compared with an American Type Culture Collection (ATCC) strain of *Bifidobacteria breve* (ATCC 15,700). All confirmation steps were biochemically based and utilize techniques familiar to the typical bacteriologist.

R. coprophilus was enumerated using a membrane concentration-resuspension, spread-plating method developed for the UMass laboratories (Arango 2000). Filtration and spread-plating are common bacteriological techniques. Appropriate volumes (10–500 ml) of environmental waters were aseptically measured and filtered through prewetted 0.2 μm absolute pore size, PVP-free (Type GTTP) membranes (Millipore Corp., Bedford, Massachusetts). The filter tower was rinsed with sterile PBS and vacuum aspirated. The membranes were then transferred into a sterile centrifuge tube containing 5 ml of Bennett's broth/pyrophosphate. The tubes were vortexed for 1 min to resuspend trapped microorganisms and then heat pasteurized at 55°C for 6–8 min to inactivate background heterotrophic organisms. Ten replicate 100 μl aliquots of each resuspension were spread plated onto MM3 media, selective for actinomycetes. The plates were inverted and incubated at 30°C for 10–14 days in a humidified incubator. The plates were then removed and placed upright at room temperature (20–28°C) and exposed to light for at least 3 days. When positive control plates inoculated with a type strain of *R. coprophilus* (NRRL-B 16537) demonstrated appropriate colony development and orange pigmentation, the sample plates were enumerated. Orange and stellate colonies without aerial hyphae were determined to be *R. coprophilus* (Rowbotham & Cross 1977; Mara & Oragui 1981). Selected confirmations were conducted by transferring a colony onto Bennett's agar and Gram staining.

Coliphages were enumerated using the membrane concentration-elution method, and plated using the double agar layer method described by Sobsey *et al.* (1990, 1995) and initially developed by Adams (1959). This method was adapted for use in the UMass laboratories (Long 1999). Phages from water samples were concentrated by adding 4 M magnesium chloride with filtration through a 90 mm, 0.45 μm (Type HA) membrane. The phages were then eluted using a beef extract-glycine-Tween 80 solution. Phages infecting *E. coli* CN-13 host were enumerated as somatic coliphages and phages infecting the *E. coli* Famp host were enumerated as F-specific coliphages. Coliphages can also be enumerated by water utilities using the Environmental Protection Agency proposed Method 1602, the single agar layer method (US EPA 2000). This method has been specifically developed for use in water utility laboratories.

For serotyping, if fewer than eight plaques were present on the phage enumeration plates, all plaques were isolated for typing. If more than eight plaques were present on the phage enumeration plates up to eight plaques were isolated per plate. For this study, all phage isolates were sent to the UNC Environmental Virology laboratory for serotyping. At UNC, they were first typed by their identity as somatic (in rare cases somatic phages will infect the *E. coli* Famp host), F + RNA or F + DNA phages. Isolates typed as F + RNA were further serotyped into Groups I, II, III or IV. Coliphage classification and serotyping experiments involve relatively straightforward spot tests described in Long *et al.* (2002).

Water quality and chemistry as well as coliform analysis were measured using standard procedures. Coliforms were analysed using Standard Methods 9222B and 9222D

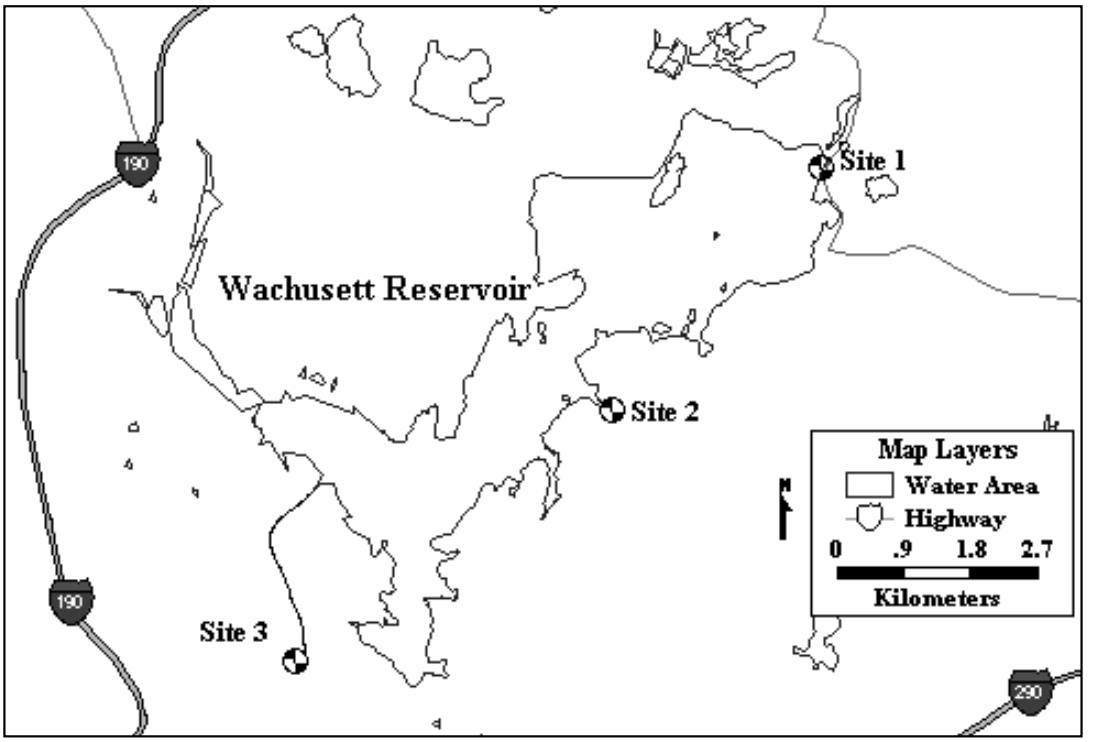

Figure 1 | Site map for Wachusett Reservoir field survey.

(Standard Methods 1998), the membrane filtration methods for measuring total coliforms (TC) and fecal coliforms (FC), respectively. Separate aliquots of each sample were filtered for TC and FC analyses. Owing to the nature of the methods (selectivity of *m*-Endo as compared with *m*-FC media) and confounding from background colonies in TC tests, in some instances final FC numbers were higher than TC numbers. Water temperature was measured using a Fisherbrand traceable water resistant thermometer (Fisher Scientific, Fair Lawn, New Jersey). pH was measured using a Corning model 430 meter (Fisher Scientific, Fair Lawn, New Jersey) calibrated to standard buffer solutions on the day of use. Turbidity was measured using a HACH model 18900 Ratio Turbidimeter, calibrated to sealed standards on the day of use. Particles were counted using a MetOne particle counter (Gilmont Instruments, Grants Pass, Oregon). Total organic carbon (TOC) was analysed on a Shimadzu TOC 5000-A analyser (Shimadzu Corporation, Australia). Each set of samples analysed included calibration standards.

RESULTS AND DISCUSSION

A map of the three field sites, Site 1 (a highly protected site), Site 2 (a wildlife impact area) and Site 3 (a residential impact area) is presented in Figure 1. The microbial indicator measurement results for each site are summarized in Figures 2, 3 and 4, respectively. Note that the frequency of detection of all microbial indicators increased from protected Site 1, to the wildlife influenced Site 2, to the human activity influenced Site 3. Microbial levels in Site 1 samples were low, as expected. Total coliforms ranged from 0 to 20 cfu 100 ml^{-1}, while fecal coliforms ranged from 0 to 15 cfu 100 ml^{-1}. The levels of

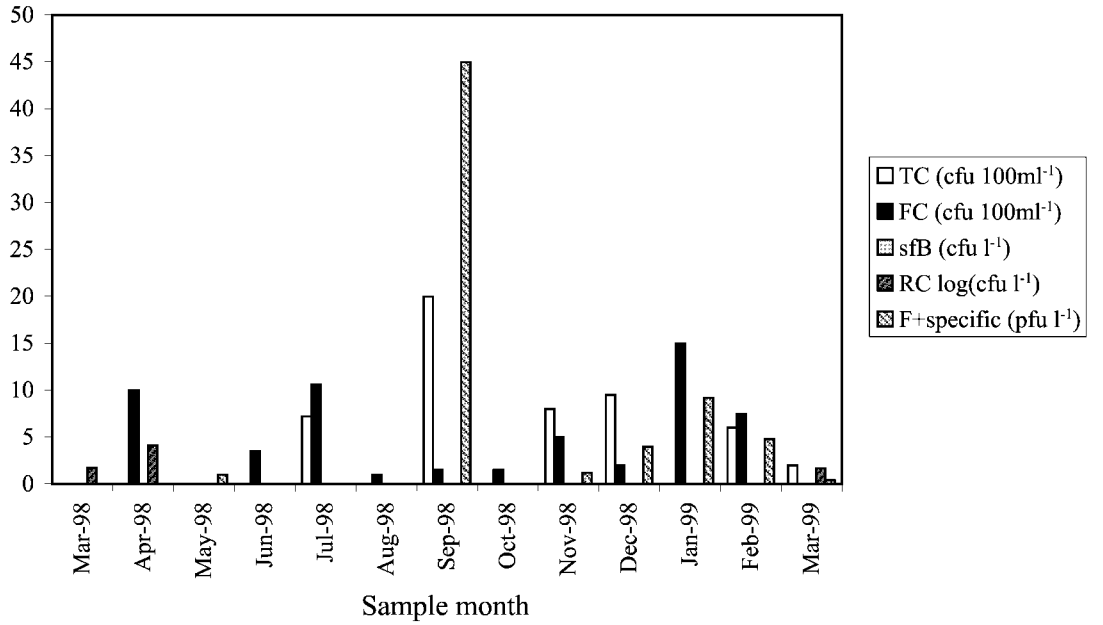

Figure 2 | Microbial survey results for Site 1.

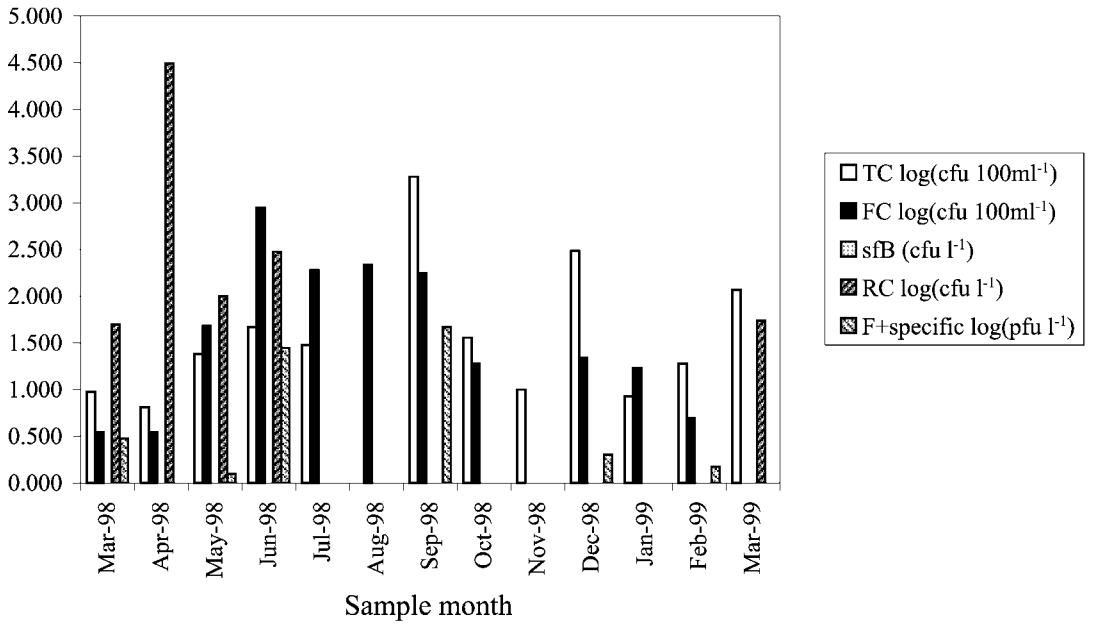

Figure 3 | Microbial survey results for Site 2.

microorganisms in Site 3 samples were the highest, with total coliforms reaching up to 2,345 cfu 100 ml^{-1}. All Site 3 samples were positive for fecal coliforms ranging from 10 to 1,730 cfu 100 ml^{-1}.

Sorbitol-fermenting *Bifidobacteria* were not detected in either Site 1 or Site 2 samples. Site 3, however, did contain sorbitol-fermenting *Bifidobacteria* in 3 out of 13 samples. The levels ranged from below detection limits

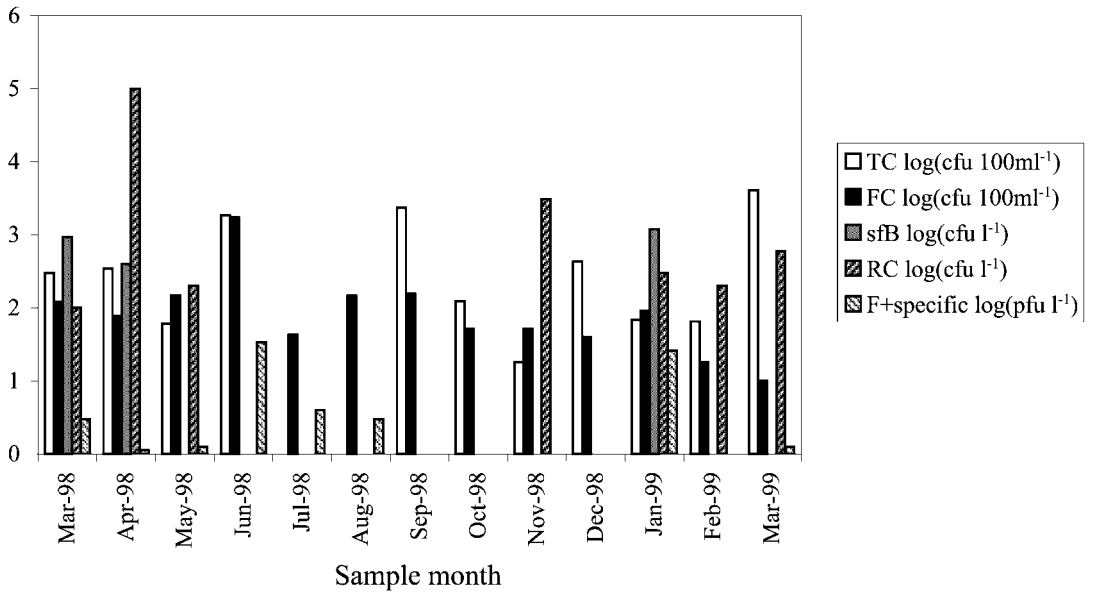

Figure 4 | Microbial survey results for Site 3.

(approximately 50–100) to 12,000 cfu 100 ml^{-1}. The presence of *Bifidobacteria* coincided with the presence of human associated serotypes (Group II) of F + RNA phages. Similarly, testing the use of sorbitol-fermenting *Bifidobacteria* as a source-specific indicator, Mara & Oragui (1985*b*) found the indicator only in waters contaminated by human feces. In South Africa, stream and river samples downstream of human settlements contained sorbitol-fermenting *Bifidobacteria* while samples upstream of the human settlements and subject to animal contamination did not (Jagals *et al.* 1995). In a study of tropical waters, the levels of *Bifidobacteria* were reported to range from 30 to 650 cfu 100 ml^{-1}; this study included one site directly impacted by sewage treatment plant effluent (Carrillo *et al.* 1985). In a study of sub-estuarine samples, levels of sorbitol-fermenting *Bifidobacteria* ranged from 1 to 90 cfu 100 ml^{-1} in tributaries subject to non-point or diffuse sources of sewage or wastewater (Rhodes & Kator 1999). Overall, the enumeration of sorbitol-fermenting *Bifidobacteria* in the Site 3 samples and absence in Site 1 and 2 samples demonstrate similar levels and trends to those reported in the literature and that the indicator can be used for temperate regions.

R. coprophilus were detected in three of the eight Site 1 samples, which were collected after rainfall events, ranging from 10 to 1,360 cfu 100 ml^{-1}. This occurrence could be attributed to the long survival time of *R. coprophilus* in surface waters allowing them to reach Site 1 (Oragui & Mara 1983). At Site 2, *R. coprophilus* was detected in 5 out of 13 samples, ranging between 65 and 3,100 cfu 100 ml^{-1}. Again, the detection was in samples collected when there had been measurable rainfall during the 24 h period preceding sampling. The presence of *R. coprophilus* at Site 2 coincided with land use data that showed a farm containing cattle approximately 1.6 km (one mile) upstream from the site. In Site 3 samples, *R. coprophilus* was detected in 7 out of 13 monthly samples, ranging between 100 and 10,000 cfu 100 ml^{-1}, again in samples collected after precipitation events. The presence of *R. coprophilus* at Site 3 coincides with land use data that shows a grazing pasture for horses draining directly into the brook less than 0.8 km (half a mile) from the site. It is interesting to note that April samples for the three sites yielded the highest levels of *R. coprophilus*. This could result from the spring thaw and rains that mobilize the winter land store and from the long survival characteristics of the indicator. These field results are consistent

Table 1 | Summary of F+coliphage serotyping results

Sample date	Site 1	Site 2	Site 3
March		18 F + DNA	9 F + DNA
April			1 F + DNA
May	2 F + DNA	1 F + DNA	1 F + DNA
June		2 Group II, 18 F + DNA	3 Group II, 31 F + DNA
July			2 Group I
August			
September	24 Group I	16 Group I	8 Group I
October			
November	3 Group I		
December	10 Group I	1 Group I	
January	19 Group I		12 Group I, 1 Group II, 1 Group I/IV
February	12 Group I	5 F + DNA	
March	1 Group I		1 Group I

with the literature. For example, in a field study conducted in South Africa, *R. coprophilus* numbers ranged from 600 to 87,300 cfu 100 ml^{-1} (Jagals *et al.* 1995), the highest levels being detected in wet weather flows in samples collected upstream of human settlements and subject to grazing animal activity.

F-specific coliphages from all three sites were serotyped and the results summarized in Table 1. Data indicated that the large majority of fecal contamination events were from non-human sources. The patterns of serogroups identified were consistent with qualitative land use evaluations. Land use patterns indicated that a probable non-human fecal source was birds. Avian sources may also have been the cause of the large number of Group I phages found at Sites 2 and 3, although land use data indicate that other animals may also be responsible. The detection of Group II and IV phages in Site 3 samples can potentially be explained by septic system leachate reaching the watercourse, while the Group II phages found at Site 2 are most likely due to an unusual event of human interference. The field results are consistent with the literature on viruses from septic systems (Scandura & Sobsey 1997) and sanitary survey information for the study sites (MDC 1995). The F-specific coliphage MS2 was reported to demonstrate little sorption or inactivation in saturated soil flow conditions (Powelson *et al.* 1990). In a comparison of the aquifer transport of a number of microorganisms, MS2 demonstrated a faster transport velocity and distance than *E. coli* or *B. subtilis* endospores (Sinton *et al.* 2000). As indicated in Table 1, a number of samples also contained F + DNA coliphages. Bird feces and wastewater are noted to contain numbers of F + DNA coliphages (Furuse 1987; Chung 1993).

Statistical analyses of water quality (data not shown) and microbial indicator data were performed using non-parametric tests in SPSS for Windows (SPSS, Inc., Chicago, Illinois). Turbidity and total organic carbon appeared to be related to the presence of fecal coliforms

at a 90% confidence level. Turbidity and particle counts were related to the presence of sorbitol-fermenting *Bifidobacteria* and *R. coprophilus* at approximately an 80% confidence level. However, none of these relationships could be used to predict another parameter. Comparisons of the traditional indicators (coliforms) to source-specific indicators revealed no statistically significant relationships.

With the widespread use of centralized sanitation in the United States, microbial contaminants from human fecal sources within a watershed, including the indicators sorbitol-fermenting *Bifidobacteria* and F-specific coliphages, result from sewer leaks and short-circuiting of septic system leach fields with rare direct deposit onto the land surface. Microbial contamination from farm and wild animal sources, and the indicators *R. coprophilus* and F-specific coliphages, usually result from direct deposit onto the land or into the water. Therefore, human sources of pathogens and indicator organisms can be hypothesized to be subject to attenuation by natural subsurface processes and reach tributaries and the reservoir through groundwater inflow and seeps from embankments. By contrast, animal sources can be hypothesized to reach tributaries and the reservoir through direct deposit of feces (e.g. roosting water fowl) and overland flow, especially associated with rainfall runoff. The source-specific indicators studied here have significantly different environmental survival characteristics from each other as well as the types of pathogens that may be present in fecal matter from the sources they indicate. Transport and survival processes must be kept in mind when interpreting indicator organism and water quality monitoring data, and caution should be used when relating a potential pathogen's presence to that of a surrogate.

CONCLUSIONS

Data collected during this field survey and laboratory analyses suggest that the three alternative source-specific indicators may be used to discriminate between different sources of fecal contamination in watershed management schemes. Sorbitol-fermenting *Bifidobacteria* can indicate the presence of human fecal contamination, and monitoring for it may show contamination in recent time and close proximity to the sampling point. This is evidenced by their detection in samples following rainfall events from the site containing residential land use served by septic systems. *R. coprophilus* can indicate the presence of grazing animal fecal contamination, and monitoring for it may show contamination for several weeks after the event. This was demonstrated by their enumeration in samples downstream from grazing animal activity after precipitation events. F + RNA coliphages are an indication of fecal contamination and can differentiate between human and non-human sources through serotyping. Animal serotypes were detected in samples from both sites at which contamination would predominate from wild or domestic animals, and human serotypes in samples from the site influenced by residential development.

Comparisons of source-specific indicator levels with coliforms demonstrate that these indicators may not be ideal for determining risk associated with drinking water sources. However, this research does support their use as watershed management tools. The frequency of positive samples for all microbial measures increased from Site 1 to Site 2 to Site 3 as would be expected from the land use and historical data for the sites. The information gathered can be used by water managers to focus watershed remediation activities in a cost-effective and efficient manner.

Analysis of the three proposed alternative indicators using the methods employed in this study can take up to 2 weeks, but the cost for the analysis is reasonable. Furthermore, the methods proposed do not require any additional laboratory instrumentation to do the tests 'in house'. The only specialized training needed is for some experience with sorbitol-fermenting *Bifidobacteria* and *R. coprophilus* colony morphology for reliable identification and production of F + RNA coliphage antisera. Analysis of the three alternative indicators can be performed at any water utility laboratory equipped for coliform analysis and standard bacteriological methods. However, more highly sophisticated molecular-based methods may be employed if they are accessible.

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