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Resazurin Reduction Tests as an Estimate of Coliform and Heterotrophic Bacterial Numbers in Environmental Samples

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Coliform bacteria are routinely used as a monitor to assess the extent of fecal contamination in surface runoff water. However, Pipes and Christian (1984) concluded that a mean coliform density cannot be reliably determined in water distribution systems using conventional membrane filter (MF) or multiple tube (MPN) techniques and a similar parallel with surface runoff water likely exists. Interferences with other heterotrophic bacteria may reduce the accuracy of conventional coliform tests. Stress factors such as low temperature and chlorine may cause these bacteria to become undetectable using conventional tests for their enumeration (e.g., LeChevallier and McFeters 1985). Another factor that acts to decrease the accuracy of microbiological water quality monitoring is the unrepresentative nature of small sample volumnes and limited monitoring frequencies. As an alternative, a coliform equivalent test using resazurin reduction has been used as a monitor of presumptive coliform levels in drinking water (Nix and Holmes 1989).

Sediment bags, which consist of sand enclosed in nylon stockings and suspended in the water column, have been used in environmental surveys to accumulate coliform bacteria over time and hence assess the extent of fecal contamination in urban streams (Nix and Merry 1990). However, laboratory analysis of the sediment bags has relied on conventional MPN techniques which are labour intensive and imprecise. As an alternative to MPN tests, a measure of dehydrogenase enzyme activity may be more appropriate in those cases where precise quantification of bacterial (e.g., coliform) levels is not required.

The concept of monitoring bacterial metabolic activity has been used for many years in the form of the resazurin reduction test for the determination of the bacteriological quality of milk (Hammer and Babel 1957). The dye is added to milk, and the rate of reduction (a colour change from blue to red) is used as a measure of bacterial contamination. The colour change can be observed visually or with increased sensitivity using a spectrophotometer at 610 nm. Resazurin reduction has also been used to determine bacterial activity in sediments (Liu and Strachan 1980).

In addition to sediment bags, the resazurin reduction test could be used as a measure of bacterial concentrations in slow sand filters used to treat raw surface runoff water for use as a source of drinking water. Present methods to enumerate bacterial cells within sand filters, and hence determine the biological "maturity" of the filter, rely extensively on direct cell count techniques. These methods may not distinguish between viable and non-viable cells (Collins and Lyne 1984) and may not be able to enumerate stressed and/or very small bacteria thought to be common in environmental samples.

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The results on work undertaken to use resazurin reduction tests as a estimate of bacterial activity in sediment is reported herein. Sand from slow sand filters was analyzed to provide an indication of the biological maturity of slow sand filters and hence their ability to remove pathogenic bacteria, as monitored by coliform tests, from drinking water supplies. In the case of water quality surveys, the test was used as a measure of presumptive fecal coliforms in sediment bags used to monitor water quality.

MATERIALS AND METHODS

A modification of the Coliform Equivalent (CE) test was used to assess the activity of presumptive coliforms in drinking water supplies. Large volumes of water (2,000 L) were filtered through sterile 0.5 micron CUNO cartridge filters. The filter and retained bacteria were incubated in a presumptive coliform medium (lauryl tryptose broth) at 35°C. Microbiological activity of coliforms and related organisms within the filter were assessed by observing the rate of resazurin reduction as compared to a standard rate for known coliform bacteria inoculated in filters in the laboratory (Nix and Holmes 1989). In this way, an estimate of presumptive coliform numbers was calculated and described as coliform equivalents (CE) per 100 mL.

The activity of heterotrophic bacteria at various depths in the sand filter was assessed by a modification of the resazurin reduction technique to measure microbial dehydrogenase enzymes (Liu and Strachan 1980). A wet weight sample (100 g) of sand was incubated in 100 mL of plate count broth (PCB) supplemented with resazurin (25 mg/L). The activity of heterotrophs was measured by incubating the sand-broth mixture at 20°C and monitoring resazurin reduction in the liquid broth at 610 nm using a Nova Spec spectrophotometer. Duplicate control test samples contained 5 mg/L $HgCl_2$ as a bactericide to indicate the contribution to resazurin reduction by chemical constituents. The difference between the rates of total and chemical resazurin reduction was expressed in umole/g/hour to provide a relative estimate of microbiological activity among the different samples. Samples of sand were collected initially when the sand filter was put into operation (March; top layer only), and in June and August surveys.

In a related experiment, resazurin reduction tests were used to estimate fecal coliform numbers in sand placed into sediment bags and exposed to stream and/or storm sewer water for 1 to 7 days. Sediment bags (Nix and Merry 1990) were tested in two different environments: an urban stream in North Vancouver (Parkside Creek); and, storm sewers in the City of White Rock, B.C. In Parkside Creek, sediment bags were suspended in the water column for 24-h periods starting on April 27, 1990 and periodically throughout the summer. At White Rock, the bags were placed on the surface of the storm sewers for a period of 7 d starting on May 15, 1990. The sediment bags (300 g of sand in a nylon stocking) were enclosed in a structured column (approx. 2 x 6 in.) made from 0.5 mm nitex mesh attached by hose clamps to large plastic bungs at either end and suspended in the water column. The sand used was a construction grade cement sand.

After exposure in the water, 100-g aliquots of sand sample were incubated in a water bath at 44.5°C in 100 mL of A-1 broth supplemented with 25 mg/L of the redox dye resazurin. Estimates of fecal coliform concentrations in sand from the sediment bags were derived by visually comparing the time for the dye to be reduced to resorufin (a pink product) with a standard curve developed by spiking 100-g aliquots of sterile sand with known concentrations of *Escherichia coli*. Since this test method was only partially selective for fecal coliforms, other bacteria might grow under these conditions, interpolated results from the standard curve were described simply as Resazurin Reduction/100 g (RR/100 g) to provide an relative indication of presumptive fecal contamination.

Levels of fecal coliforms in water samples were determined by conventional most probable number (MPN) tests using A-1 broth (APHA 1989). Due to the remote location of these sites, water samples were taken only on the day when the sediment bags were immersed into the stream or storm sewer (day 1) and then retrieved (day 2 for Parkside Creek; day 7 for the storm sewers) and an average of the two values calculated. After exposure, sand from each sediment bag was collected into sterile whirl-pac bags and fecal coliform concentrations determined using the RR test and the standard MPN test using A-1 broth (APHA 1989). MPN tests consisted of five replicate tubes with 1.0 g of test sediment added to the first series and volumetric dilutions prepared using an initial dilution of 10 g/90 mL.

RESULTS AND DISCUSSION

The resazurin reduction method was used to assess overall bacterial metabolic activity within slow sand filters as an estimate of viable bacteria concentrations. Resazurin reduction tests of the sand with the slow sand filters indicated that most bacterial activity was located in the top 1-7 mm layer or schmutzdecke compared with layers at the 300 and 600-mm depths (Table 1). This result conformed to the conventional theory of slow sand filters whereby the schmutzdecke contains the most concentrated portion of the filter in terms of biological activity. Activity was too low to be detected in March, likely due to cold spring temperatures and the fact that the filter sand had been recently installed and had not yet developed an indigenous bacterial community. Bacterial activity increased in June followed by an apparent decline in August.

Table 1. Rates of resazurin reduction in sand from various depths in a slow sand filter.

	Resazurin Reduction Rates (umoles/g/h)		
Depth (mm)	March	June	August
1 - 7	0	0.32	0.06
300	-	0.12	0.016
600	-	0	0.016

The sand filters removed presumptive coliforms at an increased rate of efficiency between March (90% removal) and June (99.9%) as demonstrated by CE test results of the water before and after filtration (Table 2). These data likely reflect the biological maturation of the filter during this period. Other researchers have concluded that biologically mature sand beds will remove greater that 99% of coliform bacteria (e.g., Bellamy et al. 1985). In August the rate of filtration efficiency decreased to 95% which corresponded to a decreased level of resazurin reduction within the sand filter.

Table 2. Coliform equivalent tests of raw and filtered water.

	Coliform Equivalent Tests (CE/100 mL)		
Month	Raw Water	Filtered Water	Reduction (%)
March	200	19	90
June	12,170	2.4	99.9
August	20,000	1,000	95
	19,500	930	

The CE test can provide a more sensitive monitor of presumptive coliform bacteria compared with conventional bacterial test methods (Nix and Holmes 1989). This increased sensitivity was reflected in high CE values (200 to 20,000/100 mL) compared with conventional total coliform counts (less than 2/100 mL) for the raw water. Although less selective for coliform bacteria compared with conventional MF or MPN tests, the CE test may be an appropriate methodology to estimate the effectiveness of slow sand filters used to remove pathogenic bacteria and/or protozoa from drinking water supplies. Coliforms are not always indicative of the presence of pathogens (Stout et al. 1984) and may not be detected as a result of the presence of other bacteria (LeChevallier and McFeters 1985) and/or the result of injury or nutrient limitations (Xu et al. 1982). In addition, small (100 mL) samples used for conventional testing may not adequately represent water quality. The test is simple and could be undertaken on-site to provide estimates of filtration effectiveness.

The concept of monitoring drinking water quality using cartridge filters to concentrate large numbers of bacteria over time has been applied to environmental monitoring using sediment bags (Nix and Merry 1990). In this case, conventional MPN tests were used to calculate fecal coliform concentrations. However, the MPN test is labour intensive and may underestimate coliform numbers due to the inability of volumetric dilution techniques to accurately dilute bacteria attached to particles.

In the laboratory, a linear regression of incubation time and cell numbers of *E. coli* inoculated into a sand substrate showed a correlation coefficient (r^2) of 0.98 indicating that cell numbers in the sediment were measurable by recording the time for resazurin to be reduced (Figure 1). At high fecal coliform concentrations, e.g., 10^9 cells/100 g, resazurin was reduced in about 1 h; however lower concentrations, e.g., 10^2 cells/100 g, required 10 - 12 h. The change in colour from blue to pink was rapid and the time required for this change could be readily estimated visually, i.e., without the use of a spectrophotometer.

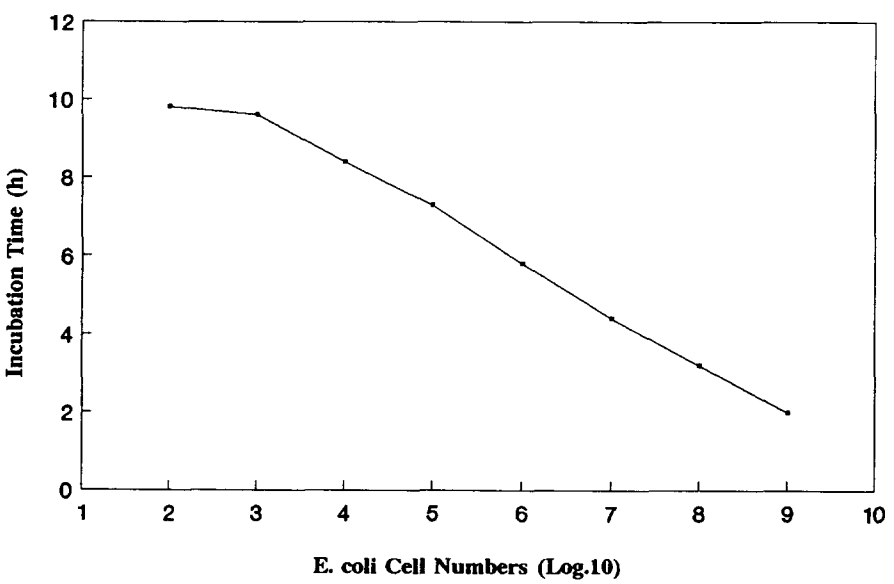

Figure 1. Enumeration of *E. coli* inoculated into a sand sediment in the laboratory using resazurin reduction.

RR test results for sediment bags placed in Parkside Creek were more sporadic compared with MPN data in terms of any coincident trends related to water quality (Figure 2). With respect to water quality, the limited data (2 samples over a 24 h period) may preclude any precise comparisons. In terms of a comparison with MPN data, the RR test may not have been as selective for fecal coliforms and/or may have been influenced by chemicals which adsorbed to the sand and either inhibited or accelerated the rate of resazurin reduction. Using replicate sediment bags, standard deviations (n = 8) for RR tests were about 100% of the mean (1,200,000 $\pm$1,400,000 RR/100 g). Standard deviations for MPN tests of the same replicate sediment bags were 5,000 $\pm$ 3,000 MPN/100 g.

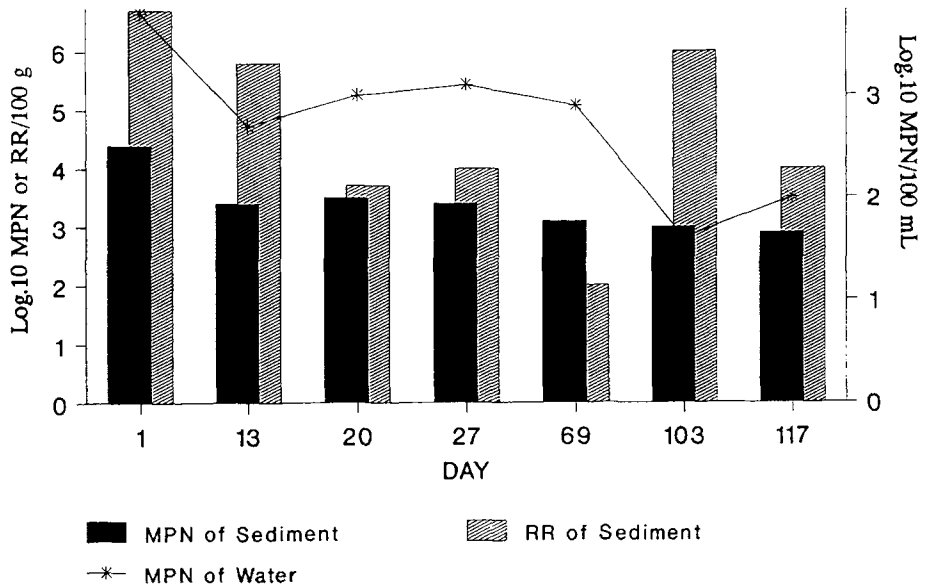

Figure 2. A comparison of fecal coliform cell numbers in sediment bags in Parkside Creek using MPN and Resazurin Reduction (RR) tests.

RR test values were generally several orders of magnitude higher compared with MPN data, a result of fundamental differences between the two tests. The RR test measures enzyme activity, not bacterial numbers. It is also less selective, since bacteria other than fecal coliforms may act to reduce resazurin. On the other hand, the MPN test likely underestimates coliform numbers due to the probable attachment of many cells onto one particle during dilution in the laboratory with the result that the particle is counted as only one cell.

In storm sewers, both RR and MPN tests of the sediment bags ranked fecal contamination at the various sites in a manner comparable with water quality data (Figure 3). To test for selectivity of the RR test, random bacteria from plate counts (R_2A agar) of the RR test supernatant were plated onto mFC medium. The majority of the bacteria isolated (83%) were identified as fecal coliforms with the exception of Site L2 (10%), indicating that, in this case, the RR test was strongly selective for fecal bacteria. Based on these results, a sanitary inspection of the area upstream from Site H-1 did show a cross-connection of the storm sewer with sewage lines.

Two subsequent surveys confirmed the ability of sediment bags to identify sites with high levels of fecal contamination using the resazurin reduction test. However, RR test results did not show the same degree of correspondence with MPN tests as during the initial survey. Storm sewer systems may have been a poor choice for this research because there are too

many uncontrolled and undocumented variables which might influence either MPN or RR test results, e.g., velocity, water depth, and/or sudden inputs of contaminants. For example, chemicals discharged into storm sewers could change redox levels which in turn could influence the chemical reduction of resazurin unrelated to bacterial numbers. In practical terms, the RR test did not act to substantially decrease analytical time requirements since results generally were apparent only after 10 to 12 h (including preparation time) compared with 20 to 24 h for the MPN test; in both cases, a period longer than normal daily working hours.

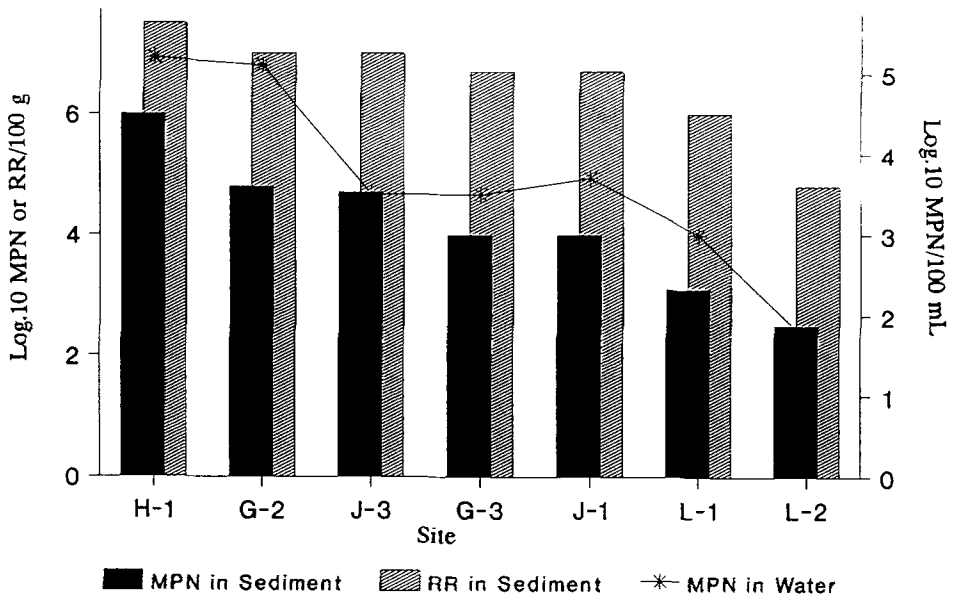

Figure 3. A comparison of fecal coliform cell numbers in sediment bags in White Rock storm sewers using MPN and Resazurin Reduction (RR) tests.

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