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Rapid selective ferrographic enumeration of bacteria

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Abstract

A low cost innovative enumeration technique that allows rapid monitoring of low bacterial concentrations is described in this paper. This technique utilizes immuno-magnetic separation methods in combination with ferrographic methods to deposit magnetic beads and attached bacteria in an exceedingly small area (0.011 cm^2) on a glass substratum. The bacteria are then enumerated under an epifluorescent microscope.

Keywords: Ferrography; Bacteria; Immuno-magnetic separation; Monitoring; Paramagnetic beads

1. Introduction

The need for improved methods to rapidly enumerate low bacterial concentrations has increased as a result of pathogen contamination of public water supplies [1–5] and increased occurrences of food-related illnesses [6,7]. Improved highly selective enumeration techniques are also necessary for transport studies of injected bacteria in groundwater. These studies are motivated by concern for pathogen contamination of water supplies, as well as by potential bioaugmentation strategies for aquifer bioremediation [8].

While methods exist for identification of many microbial strains in environmental waters, determination of the numbers of microbes that actually exist in a given sample still remains a great challenge when the microbes are present at relatively low cell counts. The ability to determine microbial concentrations is as important as identification of their genetic signatures in understanding their prevalence in environmental waters. Present methods of microbial monitoring do not allow rapid enumeration of low bacterial concentrations. Methods useful for genetic identification of microbial strains, i.e. the polymerase chain reaction (PCR), are not straightforward in quantification due to the requirement of true exponential amplification for accurate quantification [9,10]. Quantification methods such as serial dilution/culturing allow monitoring of cell concentrations as low as 1 cell/ml, but require several days to allow growth and require knowledge of appropriate substrates and growth conditions for the target microbe. Conventional filtration followed by staining and microscope identification is a rapid technique, but is not reliable at bacterial concentrations below about 10^4 cells/ml due to the difficulty of counting low numbers of cells on a filter surface (typically 25 mm diameter), and due to the confounding presence of diverse microbes in many monitored suspensions. Recently, however, the use of immuno-magnetic

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separation (IMS) of selected strains within a microbial community has been used to concentrate target strains prior to filtration. The general strategy of IMS is to tether paramagnetic beads (commercially available in sizes ranging from a few tens of nm to nearly 1 μm) to the surface of the target microbe by way of antibodies that interact specifically with the target microbe or microbes. Concentration of the target microbe can then be performed in a column of granular material which retains the magnetically tagged microbes while the column is held within a magnetic field [11–13]. Elution of the magnetically tagged microbes from the column outside of a magnetic field then provides a concentrated suspension of the target microbe. Filtration of the suspension followed by laser scanning to detect bacteria on the filter has allowed analysis of bacterial suspensions as low as 1 cell/ml [14]. However, equipment costs associated with this method may be prohibitive for many monitoring systems. This paper describes a low-cost, innovative alternative to achieve routine rapid monitoring of low bacterial concentrations.

The method described here aims at separation of microbes for visual enumeration. However, after separation, a number of steps can be taken to ensure proper identification. For example, PCR can be run on retained organisms, or organism-specific rRNA probes can be used. In contrast to laser scanning, which relies on sophisticated equipment to rapidly scan an entire filter surface, the proposed technique utilizes a strong, high gradient magnetic field to focus deposition of magnetically tagged bacteria onto an exceedingly small area ($\sim 0.011\ cm^2$) on a glass surface, allowing simple visual enumeration under an epifluorescence or conventional microscope. The method can then be modified so that viruses, which are not typically visible with fluorescent probes, can be detected by enzyme-linked immunoassays. In both cases, the results are quantitative and available in a few hours.

2. Ferrographic enumeration technique

The ferrographic enumeration technique employs a Bio-Ferrograph [15], which utilizes a magnetic field with a maximum field strength across an interpolar gap where collection of magnetically susceptible particles occurs (Fig. 1). The maximum magnetic field strength across the gap is 1.8 Tesla. However, the gradient of that field is at a maximum at the edges of the gap, thereby concentrating deposition at the gap edges. The Bio-Ferrograph directs sample volumes through a small chamber over a glass substratum on the magnetic gap, allowing efficient collection of magnetically susceptible particles. Minimal sample–apparatus contact, involving transport through about 7 cm of teflon tubing prior to deposition onto the glass slide, minimizes loss of bacteria from suspension upstream of the deposition chamber. The configuration employed allows simultaneous processing of five samples, allowing blanks to be run along with replicates or other samples.

The ferrographic technique, as related to enumeration of bacteria in natural waters, has been developed during the past four months in the environmental geochemistry laboratory of the University of Utah. Our studies have examined enumeration of *Escherichia coli* strain XL1-Blue, obtained from Dr. Joseph Holden of the Department of Pathology at the University of Utah Medical Center. The bacteria were grown in 10% tryptic soy broth to a stationary phase of 10^9–2×10^9 cells/ml. At the beginning of stationary phase, the cells were diluted into a series of suspensions ranging from 10^2 to 10^4 cells/ml with Milli-Q water containing 150 mM NaCl. Fluorescein-conjugated polyclonal rabbit antibodies (Virostat, Inc.) were attached to anti-fluorescein-coated paramagnetic beads (Miltenyi Biotec) by addition of 10 μl of stock (or diluted) antibody serum and 10 μl of stock (or diluted) bead solution to 1 ml of Milli-Q water. The solution was vortexed at lowest speed for 15 min at room temperature. Removal of antibodies unattached to beads was performed with MACS MS^+ separation columns (Miltenyi Biotec) using the manufacturer's protocol to give 0.5 ml of bead-antibody suspension. The bead-antibody suspension (100 μl) was added to 1 ml of bacteria suspension and was also vortexed at lowest speed for 15 min at room temperature. The bacteria-bead suspension was introduced to the Bio-Ferrograph at flow rates ranging from 0.001 to 0.008 ml/min.

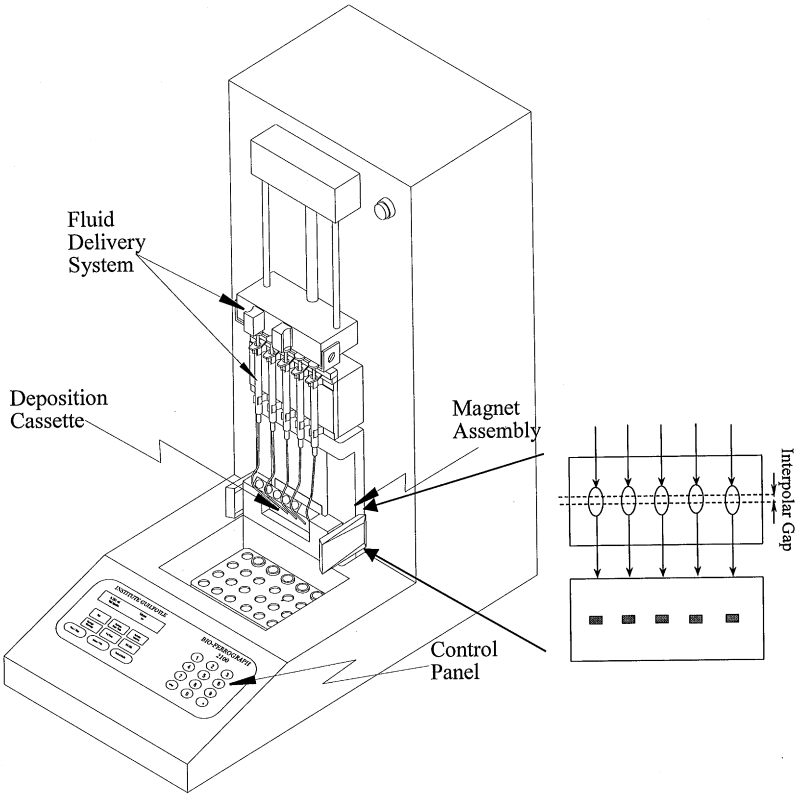

Fig. 1. The device (Bio-Ferrograph, Institute Guilfoyle) utilized in this work with schematic diagram of sample deposition on the glass substratum.

DAPI (4',6-diamidino-2-phenylindole), which non-selectively stains cells by interacting with the DNA, was introduced at the end of the run.

3. Results

The ferrographic technique deposits magnetic beads and attached bacteria in a rectangular area defined by the interpolar gap of the Bio-Ferrograph, and as illuminated by the fluorescein-conjugated antibodies under FITC (fluorescein-5-isothiocynate) epifluorescence (Fig. 2). DAPI-stained bacteria are enumerable within the area of deposition visually under DAPI epifluorescence (Figs. 3 and 4). In the case of low bacterial counts (<3000 cells per chamber), bacteria in the entire rectangular deposition perimeter can be counted (a process which takes up to about 20 min), whereas

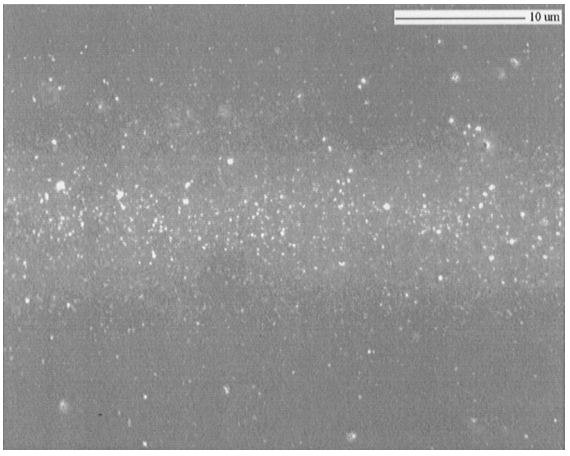

Fig. 2. Typical field of view of FITC-conjugated microbeads (no cells) deposited within the rectangular deposition perimeter under FITC epifluorescence, only a portion of the deposition area lies within the field of view. Magnification = 2000×.

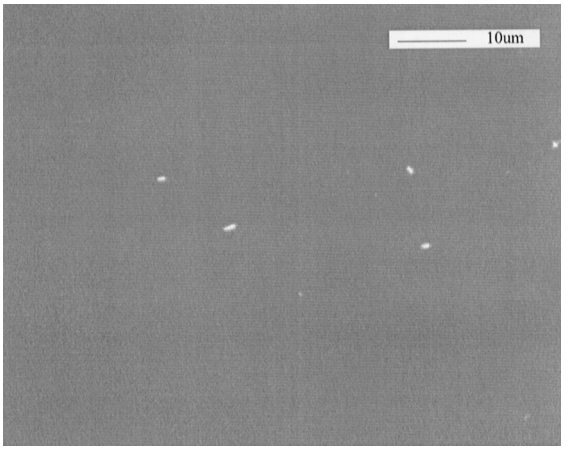

Fig. 3. Typical field of view of DAPI-stained XL1 Blue deposited within the rectangular deposition perimeter under DAPI epifluorescence. One mL of 10^2 cells/ml solution processed. Magnification = 1000×.

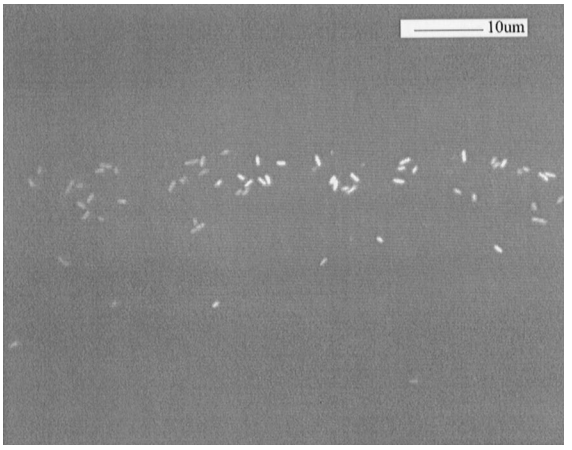

Fig. 4. Typical field of view of DAPI-stained XL1 Blue deposited within the rectangular deposition perimeter under DAPI epifluorescence. One ml of 10^3 cells/ml solution processed. Magnification = 1000×.

for higher cell counts, spot counts can be used and averaged to obtain the overall count on the rectangular deposition perimeter. The analysis can be performed using small volumes of suspension (e.g., 0.1 ml or more). Processing of larger volumes is expected to result in increased resolution, possibly to less than 10 cells/ml. The entire process can be performed within a few hours, and requires relatively simple and durable equipment.

Conventional methods of IMS have been developed for cell separation from biological fluids. Since biological fluids have cell counts typically few to many orders of magnitude greater than those found in natural waters (surface and groundwater), the bounds of successful application of the technique needed to be explored in terms of cell, bead, and antibody concentrations that yield 100% efficient capture. A series of experiments examined recovery efficiencies for various cell, bead, and antibody concentrations, as shown in Table 1. Bead and antibody concentrations were diluted to various extents relative to stock solutions received from the supplier. In Table 1 it is observed that recoveries were unaffected by flow rates up to around 0.008 ml/min, as indicated by low percent standard deviations for data averaged across these flow rates. Experiments conducted at flow rates of 0.02 and 0.05 ml/min showed ~50% and ~30% recovery, respectively (data not shown). Results from lower flow rate experiments (below 0.02 ml/min) were averaged to give average values and standard deviations for recoveries under various bead, antibody, and cell concentration conditions (Fig. 5). Fig. 5 shows that recovery efficiencies decreased as the antibody concentration was decreased for experiments utilizing high cell concentrations (10^4–2×10^4 cells/ml). This was true for experiments utilizing stock bead concentrations and 1 : 5 dilution of stock beads (Fig. 5). Equivalent results were obtained regardless of whether cells were live (culturable) or 'dead' (non-culturable), indicating that the method did not suffer from non-specific bead interaction with 'dead' cells under the conditions of the experiments (Fig. 5). In contrast, the low cell concentration experiments (100 cells/ml) show little to no effect of decreasing antibody concentration on recovery when stock bead concentrations were used, whereas recovery decreased with antibody concentration when 1 : 5 diluted bead solution was used. As was the case for the higher cell concentrations, live (culturable) and 'dead' (non-culturable) cells yielded equivalent recovery results.

Experiments were subsequently run using stock bead and antibody concentrations to enumerate

Table 1
Cell counts under microscope after ferrographic separation. The effect of flow velocity on recovery is negligible at or below 0.008 ml/min, as determined from low percent standard deviations at these flow rates. The term 'bad' refers to outlier results that are considered artifactual. Trends in observed versus expected cell counts under the different treatments are discussed in association with Fig. 5. The term 'live' refers to culturable cells, 'dead' refers to non-culturable cells

	Expected no. of cells	Antibody conc.	No. of cells recovered at given flow rate (ml/min)				Average no. of cells recovered	Standard deviation (%)
			0.001	0.002	0.004	0.008		
Dead cells	2000	Stock	1966	2092	1951	2120	2032	4.24
	2000	1 : 5	2051	1962	1982	2152	2037	4.21
	2000	1 : 10	1665	1567	1776	1677	1671	5.11
	2000	1 : 50	1368	1281	1425	1449	1381	5.41
	2000	1 : 100	942	928	1052	1033	989	6.35
Live cells	1000	Stock	945	984	908		946	4.02
	1000	1 : 5	915	941	942		933	1.64
	1000	1 : 10	797	803	777		792	1.72
	1000	1 : 50	588	569	525		561	5.76
	1000	1 : 100	bad	455	468		462	—

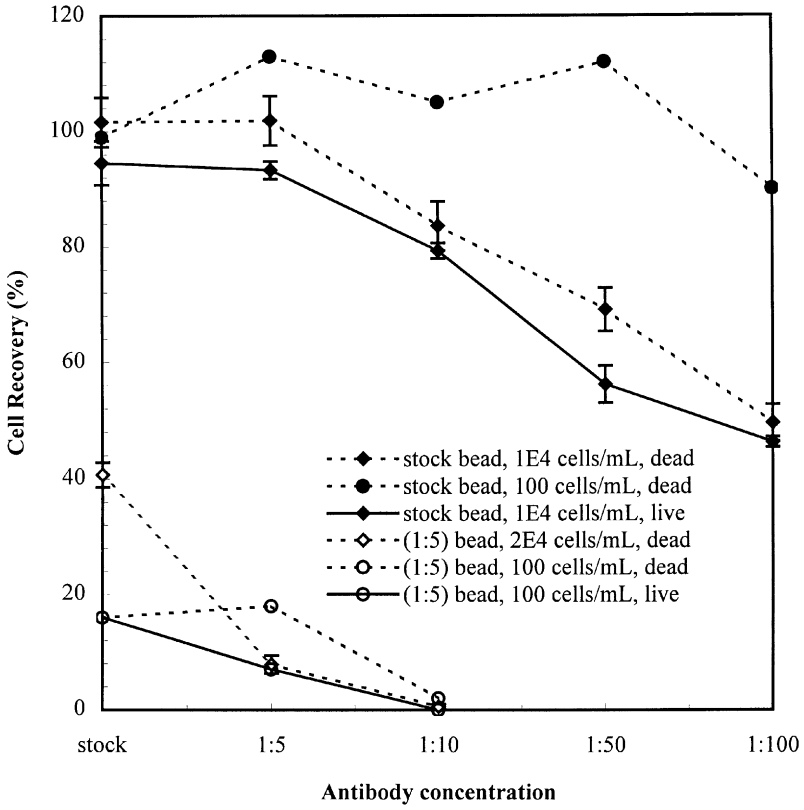

Fig. 5. Percent recoveries obtained during a series of ferrographic separations performed with varying cell count, antibody concentration, bead concentration, as well as live (culturable) versus dead (non-culturable) cells.

Table 2
Comparison of results from the ferrographic technique versus serial dilution and culturing to determine cell count in suspension ranging from 100 to 20 000 cells/ml

Estimated soln. conc. (cells/ml)	Soln. procsd (ml)	Cell counts in chamber				Ave.	Std. dev.	Std. dev. (%)	Ferrographic results (cells/ml)	Culture results (cells/ml)
		1	2	3	4					
100	1.0	138	128	134	136	134	4	3.22	134 ± 4	110
2000	0.1	236	228	186	240	223	25	11.2	2200 ± 250	2200
20000	0.1	2004	2181	1251[a]	2165	2117	98	4.63	21200 ± 980	20000

[a]Data value falls outside of 95% confidence level for average of remaining chambers and is excluded for statistical analysis.

cells in aqueous suspension (ranging from 10^2 to 2×10^4 cells/ml). The results were compared to cell counts determined by means of serial dilution and culturing (performed by Dr. Joseph Holden, Department of Pathology, University of Utah). The values between the two methods compare favorably to one another (Table 2), unfortunately, standard deviations were not available from the culturing results. However, the values substantiate the ferrographic technique as an alternate method to enumerate bacteria in suspension.

4. Discussion

The above results indicate that under the conditions of the study, use of stock bead and antibody solutions give the highest cell recoveries. Since cell concentrations vary in natural waters, it is useful to understand the principles which govern the trends observed in recovery, so that informed decisions can be made regarding optimal bead and antibody concentrations to utilize for recovery of cells at counts lower and higher than those examined in this study. The following is one explanation for the recovery trends observed here. We do not presume to present the only possible explanation for these results, but instead we present a working hypothesis to serve as a basis for decisions regarding appropriate bead and antibody concentrations to use in ferrographic separation of cells from natural waters.

The decreased recoveries achieved in the presence of decreased antibody concentrations (under high cell concentration conditions), in combination with the observation that lower fluorescence intensity accompanied decreased antibody concentrations (observed under microscope), indicates that lower antibody concentrations resulted in lower antibody coverage on the magnetic beads. Since the antibody provides the mechanism of bead attachment to the bacterium, a decreased number of antibodies per bead results in lesser numbers of potential attachment points, or lesser numbers of activated sites on the bead for attachment to the bacterium. Hence, the reduced recovery with decreased antibody concentration results from reduced numbers of activated sites on each bead, resulting in reduced bead attachment per cell during the time of the experiment. Reduced bead attachment per cell lowers the magnetic susceptibility of the bacterium to an extent that disallows 100% efficient cell recovery.

In contrast to reduction of antibody concentrations, reduction of bead concentrations from stock solution resulted in drastically reduced recoveries, even when stock antibody concentrations were used (i.e. sufficient antibodies were present to activate the beads). This indicates that insufficient beads were available per cell (even with sufficient numbers of activated sites per bead) to allow 100% efficient recovery.

It appears then, that 100% efficient separation is maintained by providing: (1) sufficient numbers of activated sites per bead to allow bead attachment to cells during the time frame of the experiment; and (2) sufficient numbers of activated beads

per cell to impart magnetic susceptibility to the cells.

The fact that the lower cell concentration (100 cells/ml) experiments did not suffer from an effect of decreased antibody concentration (at stock bead concentration), indicates that sufficient beads per cell were present in solution to make up for the lesser number of activated sites per bead. That the reduction of bead concentration by a factor of five resulted in severe decreases in recovery of low cell concentrations (100 cells/ml) regardless of antibody concentration (i.e. even with sufficient antibodies present to activate the beads) indicates that as was observed for higher cell concentrations (10^4 cells/ml), a threshold bead : cell ratio must be maintained in order to impart magnetic susceptibility to the cells.

Given that a threshold bead : cell ratio appears to be required for 100% recovery, it may at first seem inconsistent that 10^4 cells/ml concentrations with stock beads and antibodies result in full recovery, whereas 10^2 cells/ml concentrations with 1 : 5 bead and stock antibody concentrations result in poor recovery, despite the more favorable bead : cell ratio in the latter case. This apparent inconsistency can be explained by recognizing that an additional factor in recovery is the probability of bead : cell collision, which decreases as either the number of beads or cells decreases. An illustrative example of the superimposed effects of threshold bead : cell ratio and probability of bead : cell collision follows. For the purpose of this example we can arbitrarily choose the stock bead concentration to be 10^8 beads/ml. Designation of an arbitrary stock concentration was required because the actual stock bead concentration could not be obtained from the supplier. Although the actual values of bead : cell ratio and relative probability of bead : cell collision are highly dependent on this arbitrary choice, their relative values are not, so the assumed stock concentration does not affect our analysis. Table 3 shows the bead : cell ratios and the probabilities of bead : cell collision (as evaluated by multiplying the number of beads by the number of cells present in solution) in a 1 ml cell suspension with 10 μl bead solution at an assumed concentration of 10^8 beads/ml. All beads can be considered to be activated, i.e. this discussion considers only stock and 1 : 5 diluted antibody concentrations, for which it has been shown that recovery is not limited by the antibody concentration.

Table 3 indicates that the experiments which show poor recoveries are those for which either the bead : cell ratio is low, or those which show a low relative probability of bead : cell collision. It appears then that two effects govern successful recovery: (1) a stoichiometric effect which requires sufficient activated beads per cell to impart sufficient magnetic susceptibility for recovery; and (2) a mechanical effect requiring sufficient numbers of beads and cells be present in order to allow them to find one another within the relatively vast volume of solution used in these experiments. Of course, these values are representative only, since they are based on an arbitrary stock bead concentration of 10^8 beads/ml. Additional experiments would need to be performed examining varying relative probabilities of bead : cell collision while maintaining a given bead : cell ratio, and visa versa, in order to further support our hypothesis. Nevertheless, the above hypothesis provides a working basis for decisions regarding appropriate bead concentrations to

Table 3
Comparison of bead : cell ratio and relative probability of bead : cell collision (from product of bead and cell concentration) to observed recoveries under a variety of bead, cell, and antibody concentrations

Experiment	No. beads	No. cells	Bead : cell ratio	Relative probability of bead : cell collision	Observed recovery
Stock beads high cell conc.	10^6	10^4	10^2	10^{10}	100%
Stock beads low cell conc.	10^6	10^2	10^4	10^8	100%
1 : 5 beads high cell conc.	2×10^5	10^4	**20**	2×10^9	40%
1 : 5 beads low cell conc.	2×10^5	10^2	2×10^3	$\mathbf{2\times10^7}$	16%

utilize during monitoring of natural waters, and will be tested with ongoing experiments.

A major potential advantage of ferrographic enumeration over methods employing filtration is the ability to selectively collect bacteria without collection of associated mineral debris such as clays and other mineral colloids. Filtration results in deposition of mineral debris along with bacteria, potentially obfuscating the enumeration process. The ferrographic technique will not collect non-magnetically susceptible mineral debris, i.e. silica colloids and some clays [16] in the absence of non-specific magnetic bead–mineral interaction. On the other hand, magnetically susceptible mineral colloids (i.e. iron and manganese oxides and some clays), are easily removed from the sample by an initial pass through the Bio-Ferrograph prior to addition of paramagnetic beads and antibodies [16]. Subsequent experiments will examine recoveries in the presence of mineral colloids, as well as recovery of individuals from within a complex population of bacteria.

5. Conclusions

The ferrographic separation technique offers a rapid means of selectively enumerating microbes in natural waters, using inexpensive, simple, and reliable equipment. The ability to perform rapid assays is imperative for timely monitoring of drinking water and food quality. Because monitoring will necessarily need to be performed at the site in question in order to achieve rapid results, the technology used for monitoring must be inexpensive in order to be accessible to a wide variety of needs, and simple and reliable in order to be useful in less-developed regions of the globe.

An additional benefit of the method is that once the target organisms have been concentrated and enumerated, a number of confirmation steps can be taken to ensure that the identification is proper. For example, PCR can be run on the organisms retained on the glass substratum, or organism specific rRNA probes can be used. Current limitations for both of these methods generally include interfering particulate material, which is generally eliminated by the proposed procedure.

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