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ENVIRONMENTAL MICROBIOLOGY - SHORT COMMUNICATION

Separation of *Escherichia coli* from natural samples for identification of sources and microcosm inoculation

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Abstract

Obtaining uncultured *Escherichia coli* from natural waters is an important step in the study of microbes in the environment, which are critical for bacterial decay and microbial source tracking. The quality of the samples used can influence the assays, because high contaminant concentrations, differing cell ages, and physiologic states can impair results. The proposed separation is based on a three-step filtration method applied to replicates of seven samples from a sewage plant affluent, collected in different periods. Aliquots of the leachate were inoculated into microcosms, aiming to observe the cultivability of the cells. The assay resulted in colimetry values ranging between 10^4 and 10^5 cells. In the leachate, averages of 1.05% of total coliforms and 1.10% of *Escherichia coli* were recovered from original samples. Although enduring unfavorable temperatures, salinities, and nutritional conditions, the inoculated microcosm populations grew approximately 310 times after 24 h. The final leachate contained cultivable cells in appropriate physiological states and quantities for inoculum in microcosm sets. The bacteria obtained from the leachate were also appropriate for surveys of microbial source tracking, because, in the developed procedure, organisms were separated from contaminants, while cell concentrations were sufficient for inocula.

Keywords *Escherichia coli* · Wastewater · Microbial source tracking · Bacteria decay · Wild strains

Introduction

Applying cultured bacteria inoculum in vitro microbiological studies can directly influence the results of the assays, because the gene expression can be very different from natural ones. This topic is particularly critical in both (a) environmental bacterial decay studies and (b) contamination source studies (microbial source tracking, MST) [1–3]:

In decay studies, the culture of the samples alters the gene expression of the bacteria, because culture phase transitions induce pre-adaptations to osmotic shock, including variations in temperature, salinity, solar radiation, and other parameters. Thus, by applying cultured bacteria, it becomes difficult to relate results obtained from decay assays to what would actually have occurred in the environment [4–6].

As a proposed alternative to fully in vitro studies, samples can be obtained directly from the environment, which contain cells that have endured different conditions, and display several different physiological states: viable and cultivable, viable but non-cultivable, and non-viable [7–9]. However, environmental samples do not always yield cells at sufficient concentrations and viable/suitable conditions for decay studies [8–11].

Effluents from sewage treatment plants are expected to contain homogeneous bacterial populations at appropriate concentrations [12]. On the other hand, sewage has high concentrations of nutrients, solid materials, and chemical contaminants, which drastically change the growth behavior of bacterial populations. Nutrients promote intensive growth, solid

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materials can adsorb and drag down a large number of cells to sediments, and chemical contaminants may affect growth and produce matrix effects during microbial analysis [13–15]. Therefore, inoculating raw sewage water in decay experiments can impair results.

The MST studies of contamination sources use molecular markers of targeted cells [1, 3, 16], and the techniques applied for cell concentration may also lead to the chemical contaminant concentration which affects molecular analysis. On the other hand, dilution to reduce the concentrations of chemical contaminants also reduces the concentration of markers, which often hinders their detection. Besides, quantitative approaches of cells with qPCR require procedures that do not apply bioaugmentation, thereby maintaining the quantitative relationship during the analytical process [17].

The aim of this work was to develop a new technique for the separation of *Escherichia coli* from raw sewage, applying reverse filtration (RF). The procedure seeks to separate out bacteria from other contaminants in the sewage, to provide inocula with a minimum amount of contaminants and natural genetic diversity, with appropriate quality and quantity for microbiological assays.

Material and methods

Seven 150-mL samples of influent of a sewage treatment plant were obtained from the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Samples were collected in November (Spring) and December (summer) in consecutive days. The samples were collected from the diverging section of a Parshall flume, after sewage had passed through the grit chamber (before any treatment). Samples were placed in 200-mL autoclaved flasks and transported under refrigeration to the laboratory which is 100 m away from the plant, and immediately processed, according to the description in Fig. 1 (total time between collection and processing was always less than 1 h).

Considering that bacterial size does not vary considerably [18] and oscillates around 0.8 μm [19], samples were sequentially passed through 0.8-μm and 0.45-μm membranes. Samples were thoroughly homogenized and 10 mL of the samples was passed through a mixed cellulose esters 0.80-μm Millipore® membrane and the filtrate was recovered. The membrane was replaced, and the procedure was repeated until a 50-mL filtrate was obtained. Samples were continuously homogenized between steps, the filtration samples were then reserved, and membranes were discarded.

A second filtration step was performed using a 0.45-μm filter membrane from the same manufacturer. After the second filtration, the filtrate was discarded, and the membrane containing purified bacteria was reversed (top side down) in the filtration holder (as shown in Fig. 1). Then 100 mL of sterile phosphate water (following the standard methods; [20]) were passed through the filter, leaching off bacteria. A 3-mL sample of each final leachate (phosphate water) was analyzed in a spectrophotometer at 660 nm, to verify the optical density of the bacteria suspension. The remaining leachate obtained in this reverse filtration (RF) was subsequently analyzed as follows:

(a) First, analyses of the colimetry of each raw sewage influent sample were performed with enzymatic substrate Colilert® in a Quanti-Tray system. The trays were incubated at 35 °C for 24 h

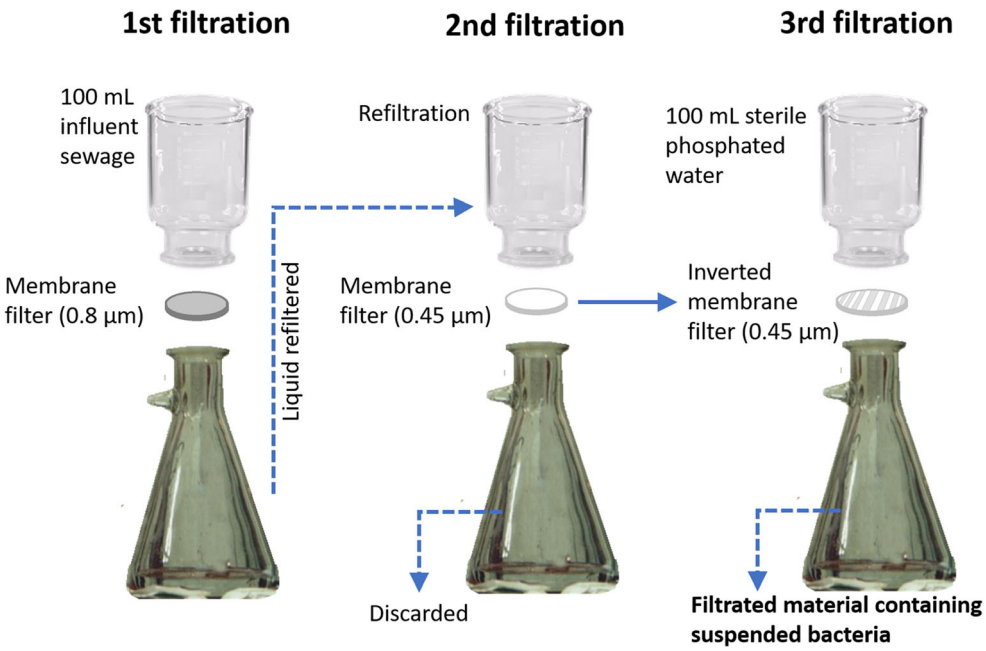

Fig. 1 Schematic design of the reverse filtration (RF) procedure. Source: authors

Table 1 Colimetry average (most probable number/100 mL) in the raw sample, after 0.80-μm filtration and after reverse filtration. Reverse filtration for each sample was performed four times

Sample (*n*)	Raw influent		Control after filtration in 0.80 μm		After reverse filtration in 0.45 μm (4 replicates)	
	Total coliforms	*E. coli*	Total coliforms	*E. coli*	Total coliforms	*E. coli*
1	4.9E+07	9.6E+06	2.5E+06	5.4E+05	4.4E + -5	1.1E+05
2	2.4E+07	8.8E+06			1.8E+05	8.3E+04
3	5.7E+06	2.3E+06			7.1E+04	3.3E+04
4	5.7E+06	2.3E+06			5.2E+04	2.5E+04
5	1.6E+07	6.5E+06	1.9E+05	8.2E+04	1.8E+05	6.3E+04
6	1.4E+07	7.3E+06			1.8E+05	7.0E+04
7	1.3E+07	7.7E+06			1.9E+05	7.8E+04

(b) Replicate colimetry of the phosphate waters (which leached 0.45-μm filters) was carried out with the same Colilert® procedure. Two subsamples of phase 1 filtrate from samples 2 and 5 were carried out, with the aim of controlling whether the filtrations at 0.80 μm were unexpectedly retaining bacteria.

(c) A second aliquot of the leachate was used to inoculate microcosms in the laboratory. In total, 750 mL of sterile phosphate water received 26.25 g of sterile NaCl, yielding a solution of 3.5% (simulating seawater) and 0.75 mL (0.1%) of Brain Heart Infusion broth. The solution was inoculated with 7.5 mL of leachate obtained from the reverse filtration. The experimental setting was divided into three 250-mL portions and transferred to a 500-mL Erlenmeyer flask, constituting triplicates with the simulated seawater.

The above-described microcosms were incubated in a 60 RPM shaker at 20 °C (room temperature, simulating natural environmental conditions) for 24 h. After this incubation period, colimetry was made as described previously.

Results and discussion

The primary results of the reverse filtration tests are presented in Table 1, where it can be observed that in all tests, total coliforms (TC) and *Escherichia coli* (EC) were obtained in the final leachate with the proposed reverse filtration (RF), presenting concentrations ranging from 10^4 to 10^5.

The percentages of cells recovery shown in Table 2 ranged from 0.77% (in sample 2) of total coliforms (average 1.05 ± 0.31%) to 1.46% (in sample 3) of *E. coli* (average 1.10 ± 0.37%). Comparatively, the recovery after the first filtration yielded 5.04% and 5.64% of total coliforms and *E. coli* (sample 1) and 1.21% and 1.26% (sample 5), respectively. Thus, it is pertinent to consider that a large proportion of the bacteria were adsorbed to the solid materials, which were retained in the 0.80-μm pore filter used in the initial stage. The filtration through 0.45 μm also eliminated most of the dissolved materials as indicated by spectrometric absorbance.

Although the percentage of cell recovery did not correlate with the initial concentration ($r^2 = 0.223$ and 0.384 for total coliforms and *E. coli* respectively), it was observed that the recovery approximately agreed with the decay pattern. However, there was a linear correlation between the initial and final absolute values, both for total coliforms ($r^2 = 0.936$) and for *E. coli* ($r^2 = 0.938$), as can be seen from Fig. 2.

The reverse filtration method (RF) generates a translucent final leachate with an absorbance of 0.00 (at 660 nm), against milky phosphate water used as blank control. It can be observed from Table 3 that the *E. coli* population from the RF inoculum was able to grow even when the microcosm was saline and supplemented with only 0.1% Brain Heart Infusion (BHI) broth.

Table 2 Colimetric yield after reverse filtration (RF) and percentages of the RF leachate colimetry in relation to raw sewage

Sample	Total coliforms (% in the filtrate)	*Escherichia coli* (% in the leachate)
1	0.89 ± 0.09	1.15 ± 0.39
2	0.77 ± 0.11	0.94 ± 0.25
3	1.26 ± 0.35	1.46 ± 0.38
4	0.92 ± 0.23	1.11 ± 0.51
5	1.13 ± 0.02	0.97 ± 0.02
6	1.19 ± 0.25	0.96 ± 0.04
7	1.46 ± 0.42	1.01 ± 0.17
Average	1.05 ± 0.31	1.10 ± 0.37

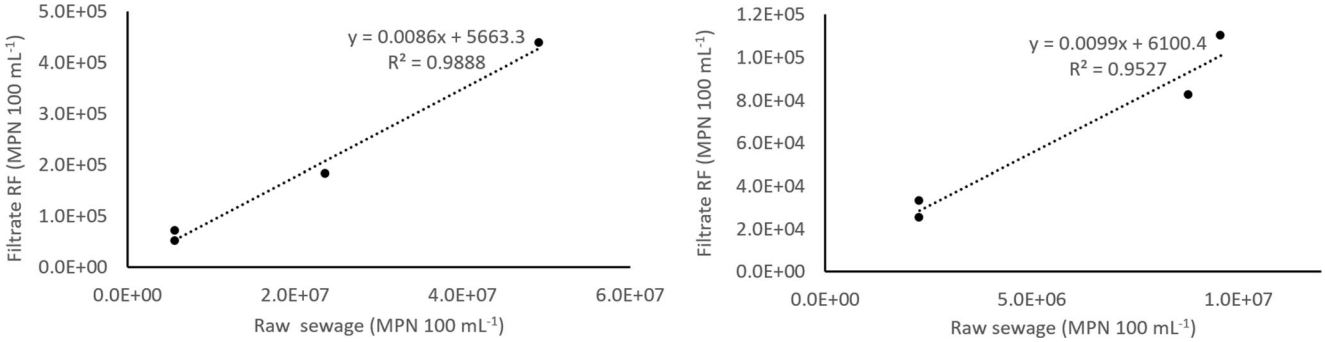

Fig. 2 Correlation between total coliform (left) and *Escherichia coli* (right) in raw sewage and yield in the final filtrate (reverse filtration)

Purified *E. coli* obtained from reverse filtration for inoculation in microcosm decay assays presents appropriate concentrations and physiologic conditions, but does not require the cultivation of natural samples. In microbial source tracking (MST) approaches for water quality surveys, it is desirable to use samples with little chemical contaminants (frequently present on the raw water) that may constitute interferences in the analytical procedures. The proposed reverse filtration (RF) was shown to be a fast, easy, and practical procedure for the separation of bacteria from raw water. It can be processed in approximately 60 min, producing a leachate with natural wild bacteria that fulfill the needed requirements, but stripped of water contaminants from where it was collected.

The 99% reduction on cell count, when compared with the original sample, was associated with adsorption to abundant solids in the sewage, but this value can be significantly reduced when the procedure is applied to natural waters (instead of raw sewage, as in the present research). Despite this reduction in cell count, the leachate obtained from reverse filtration proved to be adequate for *E. coli* inoculation in microcosms, because the inoculum contained enough cells to repopulate the microcosm. Besides, the bacterial inoculum constituted only a small fraction of the microcosm volume, without adding solid materials to the experimental set, as measured turbidity was insignificant. Particles between 0.45 and 0.80 μm were retained in the membrane and were leached together with bacteria in 100 mL of phosphate water, explaining the low absorbance (at 660 nm). In this case, residues that could be ecotoxic to the bacteria were adsorbed in the membrane and were significantly diluted during the last step, with only a small portion reaching the final leachate. Moreover, a leachate volume corresponding to only 1% of microcosms was used to inoculate, a dilution factor of 100.

Finally, a comparison of the presented method with current procedures applied in the literature highlights their advantages and pitfalls. Table 4 summarizes the advantages of reverse filtration in relation to preparation procedures currently used for inoculation in microcosms or environmental molecular evaluations. Inoculation of raw sewage [2] or raw natural water [21] shows pitfalls like varying physiological state, contamination with chemicals and nutrients that modify bacterial reproduction. Cultured bacteria as applied in the works of Jozic et al. [5] and Eichmiller et al. [22] may not respond adequately to natural processes, because these organisms display small genetic variation.

Although bioaugmentation [23] allows an increase in detection levels in molecular analyses for water quality evaluation, quantitative pieces of information are lost. On the other hand, cell concentration procedures [24] improve detection, but contaminants are also concentrated and may interfere with the results. Molecular analyses applying nucleic extractions also have been used to evaluate the resistance of bacteria under natural conditions [25]. However, the loss of molecular targets is expected.

Conclusion

The final leachate (from reverse filtration) contained viable and cultivable *E. coli* cells, in physiologically suitable conditions and quantity to produce inocula for microcosm tests. The results of the microcosm obtained after 24-h inoculation confirm this statement, when the inoculated population had grown approximately 310 times, although under unfavorable temperature, salinity, and nutritional conditions.

Table 3 Comparison of cultivable coliforms (most probable number 100 mL^{-1}) obtained in reverse filtration (RF) after 24 h of incubation in the microcosm (dark conditions and 3.5% NaCl)

Sample 2	Conc. filtrate RF	Concentration inoculum	% BHI added	24-h microcosm	Replicates
Total coliforms	1.8E+05	1.8E+03	0.1	8.2E+06	3
E. coli	8.3E+04	8.3E+02	0.1	2.6E+05	3

Table 4 Comparison of current preparation of samples for inoculation in microcosms or environmental molecular evaluations with reverse filtration (RF)

Type of study	Procedures from the literature	Negative aspects	Advantages of reverse filtration
Inoculation of microcosm or mesocosm	Inoculation with raw natural water or sewage [2, 21]	Bacteria have varying cellular and physiological states. Various generations may be present in the same sample Presence of chemical contaminants, excess nutrient, and solid materials	Age and physiological state of the cells remain homogeneous. Cells are viable and cultivable Inoculation can be done with freshly sampled raw sewage with no cultivation, minimizing dissolved contaminants and nutrients
	Inoculation with cultured bacteria [5, 22]	Alteration of the genetic expression of bacteria.	
Molecular analysis of natural waters quality	Bioaugmentation (pre-enrichment) [23]	Quantitative approach is invalidated.	No bioaugmentation is necessary, reducing time spent. A qualitative approach is possible.
	Cell concentration [23, 24]	The procedure of cell concentration increases the amount of dissolved contaminants that damage genetic materials for PCR analyses	Cells remain viable and can be quantified by PMA-qPCR. Concentration of cells can be carried out together with purification, reducing dissolved contaminants and minimizing damages to the genetic material to be extracted.
	Nucleic acid extraction and purification [24, 25].	The nucleic acid extraction and purification result in the loss of molecular targets.	

The results presented here allow to conclude that the leachate obtained by reverse filtration is also appropriate for microbial source tracking approaches, because contaminants in the sample were stripped off, while yielding cell concentrations compatible with what is usually required for this kind of studies. Furthermore, the obtained cells were shown viable and the leachate can be submitted to quantitative analyses of PMA-qPCR [26].

From the presented procedure, there is no need for culturing samples to obtain cells for inoculum, nor for augmenting the number of cells for molecular biochemistry surveys. The possibilities of alterations in genotypic expression—a frequent pitfall during cultures, such as those that occur in the transition from the log phase to the stationary phase—are reduced. Further studies are needed, mainly related to the chemical composition of the final leachate and the genetic expressions of the produced bacteria.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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