An Improved Spectrophotometric Method To Study the Transport, Attachment, and Breakthrough of Bacteria through Porous Media

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This study reports an improved spectrophotometric method for studying bacterial (Pseudomonas fluorescens UPPer-1) transport and attachment in saturated porous media (silica sand). While studying the effect of ionic strength by the traditional packed-column spectrophotometric method, we encountered an artifact. The absorbance of a well-stirred bacterial suspension was found to decrease with time in the presence of high concentrations of sodium and potassium phosphate salts (≥10⁻² M) as the cells continued to age in a resting stage. Our results show that collision efficiency and a bed ripening index will be in error by as much as 20% if breakthrough is measured by the traditional spectrophotometric technique. We present an improved experimental technique that will minimize the artifact and should substantially advance the understanding of bacteria transport in porous media.

Bacterial transport through saturated porous media has received increased attention in the past decade, spurred by the need to understand and engineer subsurface bioremediation. In particular, bioaugmentation involves injecting specific strains of bacteria into the subsurface. The success of this approach requires that the bacteria be transported to the zone of contamination and attach to the solid matrix in a controlled fashion. Prior information about the effect of geochemistry on bacterial transport and attachment is therefore very important.

Using the terminology from the filtration of inorganic colloids in porous media, bacterial transport may be characterized by two parameters, collector efficiency (η) and collision efficiency (α). Collector efficiency is defined as the fraction of approaching colloids which strike a collector and collision efficiency is defined as the fraction of colliding particles which are successful in attaching to the collector. Using the packed-bed technique (4), bacteria can be passed through a porous medium packed column and the effluent concentration (C/Co) as defined below) can be monitored with respect to time. Accurate measurements of these data (breakthrough data) are essential to correctly obtain the values of collision efficiency as seen from the following equation for a deep-bed filter (4):

\[ \alpha = 4 \frac{a_c}{3(1-\theta)\eta L} \ln \left( \frac{C}{C_o} \right) \]  

In equation 1, ac is the radius of a collector (meters), θ is the bed porosity, L is the bed length (meters), and C and C0 are effluent and influent concentrations of cells (cells per milliliter), respectively. Thus, errors in the breakthrough data (C/Co) will have a logarithmic effect on the calculated values of α.

Examples of experimental techniques to study the deposition of cells and other colloids in porous media are (i) packed-bed technique (3, 4, 5, 7, 9, 18), (ii) stagnation point flow technique (4), (iii) rotating disk system (17), and (iv) parallel-plate channel technique (4); however, the packed-bed technique has been the most widely used. The influence of various physical and chemical factors on microbial transport through packed-bed porous media was studied by Fontes et al. (5). Bacteria were found to be retained more on the porous media at high ionic strength (0.0089 M) compared to a lower ionic strength (0.00089 M) of artificial groundwater. A packed-column technique involving radiolabeled cells which gives a direct measurement of bacterial attachment in packed-column sections has been reported (7, 9). Use of a rotating disk system in quantifying the bacterial collision efficiency has also been reported (17), which also pointed out the increase in bacterial collision efficiency with the increase in ionic strength. McCaulou et al. described a short-pulse technique to calculate bacterial collision efficiencies in packed-bed columns, used to study the collision efficiencies of hydrophilic and hydrophobic bacteria (18).

The concentration of the bacteria in the effluent stream of a packed-bed column can be measured with either of the following techniques: (i) counting of CFU (2, 10, 23), (ii) acridine orange direct microscopic counting (8), (iii) radiolabeling of cells followed by scintillation counting (13, 14, 16), and (iv) spectrophotometric analysis (3, 4). Microscopic counting is tedious, prone to the researcher’s judgment, and not amenable to a large number of samples. In addition, there are safety concerns associated with the use of radioactive labeling.

The use of a spectrophotometric technique is not new to the study of transport in porous media of both colloids (20, 12) and bacteria (21) or for the study of bacterial adhesion to host components of cells and tissues (22). When this technique is used to investigate the effects of solution properties such as ionic strength, it is important to minimize or eliminate experimental artifacts which will interfere with the light absorbance properties of the suspension. The aim of this study is to present an improved method which is demonstrated to be less prone to such artifacts. The proposed method addresses these concerns by building on an experimental methodology suggested for inorganic colloids (4).

The traditional method for studying bacterial transport in porous medium columns involves the use of a packed-bed technique. A schematic diagram of this experimental setup is shown in Fig. 1. The system typically includes a feeding reser-
voir containing the bacterial suspension and the salt solution (5, 7, 9, 16, 21), a metering or peristaltic pump to convey the suspension, and a column packed with the granular porous medium. A surge bottle is sometimes added after the peristaltic pump to obtain a smooth flow.

The absorbance of the bacterial suspension, measured with a spectrophotometer, is assumed to be a constant value for the inlet to the column for the entire experiment. The experiment is then started by switching the inlet flow to the bacterial suspension. Effluent bacterial absorbance is spectrophotometrically monitored with respect to time. The ratio of the inlet to the outlet absorbance at any time is used to construct the relative concentration breakthrough curve ($C/C_o$).

Absorbance ($A$) is an indirect measure of the bacterial concentration in a suspension. It is directly related to turbidity ($\tau$) by

$$A = l \tau$$

where $l$ is the path length of the suspension (6). Turbidity is further defined as

$$\tau = N C_s$$

where $N$ is the number concentration of the bacteria and $C_s$ is the scattering cross section of the bacteria. $C_s$ depends on the size and refractive index of the bacteria and on the wavelength of the incident light (6). As the size of the bacteria decreases well below the wavelength of light, values of the scattering coefficient become close to zero, resulting in a decrease in the turbidity.

Operating in the linear range of the spectrophotometer provides a direct correlation between the absorbance values and bacterial concentration. Absorbance measurements can therefore be directly converted to cells per milliliter using a calibration curve. It is very convenient to operate a spectrophotometer with a low-volume flowthrough cell, which allows continuous monitoring of the bacterial concentration.

**Materials and Methods**

**Growth of microorganisms.** Because the conditions for cell growth (pH, ionic strength, etc.) and transport may be very different, there are two main approaches in conducting these experiments. One approach allows the cells to adapt to the transition from bioreactor to experimental conditions, i.e., allows cells to undergo natural changes to cellular properties. The main advantage is that the resulting transport characteristics are more reflective of the evolved properties. The disadvantage is that the resulting transport characteristics are not necessarily responding to the manipulated experimental variable (ionic strength in this work) but rather are influenced by cellular properties in ways that are not well understood and may vary with time. Another approach introduces the cells to the experimental conditions (different ionic strengths in this work) very quickly prior to the experiment, thus eliminating adaptation. The advantages and disadvantages in this latter case are just the opposite compared to the prior approach. We have adopted the latter approach in this work because our primary interest is in understanding the influences of ionic strength (the introduction of cations and anions) on bacterial transport and attachment in porous media. The influence of adapted cellular properties on bacterial transport is obviously an important area of investigation as well but was not a part of this work.

The bacterium used in these experiments was *Pseudomonas fluorescens* UPER-1, a rod-shaped, gram-negative microorganism. More details about the bacterium, growth media, and growth conditions in the bioreactor can be found elsewhere (3). The growth conditions were maintained constant in the bioreactor so that the bacterial properties did not change from experiment to experiment. At the harvesting stage, the bacteria were pumped out of the bioreactor and 960 ml of the cells was centrifuged in a Marathon 22 K centrifuge (Fisher Scientific) at 11,000 rpm (13,900 $\times g$) for 5 min. The cells were resuspended in deionized water and spun down two more times to wash the medium off the cells. The concentrated cell suspension was then added to approximately 2 liters of deionized water in a glass beaker until the $A_{540}$ of the cell suspension became approximately 0.35. This value of absorbance is equivalent to $3.6 \times 10^7$ cells/ml, as determined using phase-contrast microscopy and a counting chamber (15). This suspension was constantly stirred for the entire duration of the column transport experiments to keep the cell suspension well mixed.

**Improved spectrophotometric method.** The sand used in these studies was coarse silica sand (Asgco Corp.) having a hydraulic mean diameter of 740 µm (company literature). The sand was cleaned with the aid of only deionized water, and no harsh reagents were used. Details concerning the sand cleaning and packing procedures can be found elsewhere (3). The column used in the studies was a glass chromatography column (Adjusta-Chrom; Ace Glass Inc.) with an internal diameter of 2.5 cm. The column was always packed to a settled bed height of 30 cm in such a way as to minimize the trapping of air bubbles (3). A schematic diagram of the experimental setup (Fig. 2) shows two separate reservoirs for the bacterial suspension and the salt solution. The salt solution was an equimolar mixture of sodium phosphate (dibasic) ($Na_2HPO_4$) and potassium phosphate (dibasic) ($K_2HPO_4$). Before the bacterial injection was started, the salt solution with no bacteria was passed through the column for 2 h at twice the flow rate used in the experiment. This was found to be sufficient to stabilize the pH of the sand and to flush out residual fine particles. The bacterial suspension was then pumped into the column at a constant flow rate of 5.0 $\pm$ 0.3 ml/min (interstitial velocity = 2.6 cm/min). The bacterial suspension was pumped using a metering pump (model OG150; Fluid Metering Inc.). Another peristaltic pump (Minipuls 3; Gilson Inc.) was used to add concentrated salt solution to be mixed in line with the bacterial suspension, creating the desired salt concentration. Four contraction-expansion joints were provided to promote in-line mixing, the effectiveness of which was verified in separate phenol red dye injection tests. Two spectrophotometers were connected in-line, one at the inlet and the other at the outlet of the column, to monitor the absorbance of bacterial suspensions. Absorbance data can therefore be easily converted into breakthrough data by the following relationship:

$$\frac{C}{C_o} = \frac{A_{540}}{A_{540,0}}$$

A personal computer equipped with data acquisition software (LabView; National Instruments) recorded the spectrophotometer absorbance values at the inlet as well as at the outlet of the column at fixed time intervals of 30 s during breakthrough and elution and for every 5 min during the rest of the experiment. The breakthrough data were subsequently normalized to obtain relative bacterial concentration ($C/C_o$) versus pore volume. The experiment was continued until the desired pore volumes (approximately 25 pore volumes) of the bacteria were passed through the column. The bacterial injection was then stopped and the elution was started using a cell-free salt solution of identical concentration. It was continued until the $A_{540}$ reached a low value of $<0.005$. The glass containers at...
RESULTS AND DISCUSSION

The first results to be presented illustrate the experimental artifacts obtained with the traditional packed-bed technique. Figure 3 illustrates the effect of phosphate salt concentrations on the bacterial breakthrough curves when the breakthrough data are obtained by the traditional packed-bed technique shown in Fig. 1. Only the effluent concentration data were monitored, and the breakthrough data were obtained by assuming a constant influent concentration. The phosphate salts were dissolved in an inlet reservoir in which the bacterial suspension was being stirred for the entire duration of the experiment. The breakthrough curves show a significant dependence on the concentration of phosphate dissolved in the bacterial suspension. As the concentration of the phosphates increases from zero to $10^{-1}$ M, the breakthrough curves progressively achieve smaller values, indicating greater attachment of bacteria to the porous media. To confirm our assumption of a constant value of $C_{in}$, we checked the bacterial concentration in the inlet beaker with a spectrophotometer. This concentration was also found to decrease with time.

To investigate this decrease in the influent cell concentration, we performed an experiment in which the bacteria were suspended in deionized water in the same beaker and at the same volume, concentration, and stirring speed as used in the column transport experiments. A flowthrough cell inserted within a visible spectrophotometer was connected to the cell suspension. The metering pump used in the column transport studies was used to pump the bacteria through the flowthrough cell at the same flow rate ($5.0 \pm 0.3$ ml/min). Phosphate salts corresponding to a concentration of $10^{-2}$ M were added to the stirred cell suspension at time zero, and the absorbance of the cell suspension was monitored with respect to time. The data are shown in Fig. 4. As seen from the graph, the absorbance begins to decrease with time after about 1 h and then remains constant after about 4 h. This shows the effect of high phosphate concentration on the light absorbance properties of the cell suspension as the cells continue to age in the resting state in the presence of the salt. This experiment points out the importance of mixing the salts with the cell suspension in-line rather than in the beaker. This is done to minimize the amount of time that the cells are exposed to high salt concentrations and the potential for cell aggregation or osmotic stress.

To further investigate the contribution of this time-dependent decrease in the influent cell absorbance to the observed decrease in the column effluent cell absorbance, a control experiment was carried out. The experimental assembly used for the control experiment was similar to that shown in Fig. 2 in all respects, except that no sand was packed in the column. A bacterial suspension was passed through a 30-cm-long empty column, and a $10^{-2}$ M phosphate salt solution was created using in-line mixing. Both the inlet and outlet concentrations were monitored as proposed in our improved method, and breakthrough data were collected. The experiment was performed in duplicate, and the average data are shown in Fig. 5. The figure does not show a significant decrease in the relative outlet concentration ($C/C_o$) with respect to time. This data indicate that the sand column breakthrough data obtained by using our method (Fig. 6) are not affected by any artificial decrease in the cell absorbance properties, as the cells travel through the sand column. Rather, the observed breakthrough curves are a result of cell attachment to the porous medium packed in the column.

Another set of experiments was conducted using the new experimental assembly and procedure. In Fig. 6, bacterial breakthrough curves are again shown as a function of phosphate concentration. There is a significant difference between the two data sets (Fig. 3 and 6), especially at higher phosphate concentrations. Data in Fig. 6 show lower bacterial attachment (higher breakthrough curves) than data in Fig. 3, even though the experiments were conducted under similar conditions of ionic strength but using different spectrophotometric monitoring methods. Further, the magnitude of collision efficiency ($\alpha$) is dependent on the height of the breakthrough curve as dis-

FIG. 3. Effect of phosphate salt concentration on bacterial breakthrough curves; data obtained by traditional packed-bed technique. DI, deionized.

FIG. 4. Effect of $10^{-2}$ M phosphate concentration on the absorbance of a well-stirred bacterial suspension measured using a flowthrough cell. Data for bacterial suspension in deionized water are shown in Fig. 11.

FIG. 5. Control (empty) column experiment indicating insignificant decrease in $C/C_o$ at a phosphate concentration of $10^{-2}$ M.
cussed in the introduction (equation 1). Thus, the two methods will yield different values of $\alpha$ under identical conditions.

The breakthrough curves in Fig. 6 are shown for each salt concentration in Fig. 7 to 11 along with the influent and effluent bacterial suspension absorbance data. A bacterial breakthrough curve at a phosphate concentration of $3 \times 10^{-2}$ M is shown in Fig. 7. A breakthrough curve referred to as uncorrected $C/C_o$ is calculated by assuming a constant $C_o$ with respect to time. The figure points out a decrease in the inlet absorbance ($A_{500,0}$) with time despite the use of in-line mixing of salts, a procedure meant to minimize this effect. However, the effect of this decrease in $A_{500,0}$ over time is minimized in the new setup compared to the traditional packed-bed technique, which would have led to the markedly different breakthrough data shown as the uncorrected $C/C_o$. The new method for collecting breakthrough data takes into account the dynamic values of $A_{500}$ and $A_{500,0}$ and leads to more accurate breakthrough data.

Figure 8 also shows a slight decrease in cell absorbance at the inlet at a salt concentration of $10^{-2}$ M. However, the importance of monitoring both the inlet and outlet concentrations is again evident from the different breakthrough curves designated $C/C_o$ and uncorrected $C/C_o$. Figures 9 to 11 show data for phosphate concentrations of $10^{-3}$ and $10^{-4}$ M and for deionized water, respectively. The difference between the $C/C_o$ and uncorrected $C/C_o$ breakthrough curve is slight or negligible for these dilute salt concentrations.

Figures 7 to 11 demonstrate that the impact of the salts on the aging resting-state cells is important at higher salt concentrations ($>10^{-3}$ M). The decrease in the absorbance of the influent bacteria was not observed for lower salt concentra-

tions of $10^{-3}$ M (Fig. 9) and $10^{-4}$ M (Fig. 10) and when the bacteria were suspended in deionized water (Fig. 11). This further indicates that stirring alone is not the reason for decrease in bacterial absorbance but stirring for longer times and subjecting cells to high salt concentrations is. Mixing of salts in-line for the purpose of minimizing inlet absorbance changes has been previously suggested as part of a packed-bed technique for deposition kinetics of inorganic colloids (4). However, for bacterial systems, the assumption of constant inlet particle concentration is not valid at high salt concentrations even with this configuration. Thus, in-line mixing as well as continuous monitoring of the cell concentration entering the column is essential.

Our results show clearly that only at unnaturally high ionic strengths would the artifacts be a problem. However, such high ionic strengths might be encountered during engineered bioremediation where the presence of inorganic nutrients might be high enough to induce these artifacts. Long-term experiments requiring long-term contact between the salt and the bacteria indeed would be very useful, and our results show the precautions that researchers should take before conducting such experiments.

Change in bacterial transport and attachment model parameters due to the improved method. The transport and attachment of bacteria in the porous media can be mathematically described by using an advection-dispersion equation coupled with rate equations, which relate to the attachment and detachment of bacteria to and from the porous media. A set of such mathematical equations which simulate the bac-

![Figure 6](image6.png)

**FIG. 6.** Effect of phosphate salt concentration on bacterial breakthrough curves; data obtained by modified packed-bed technique. DI, deionized.

![Figure 7](image7.png)

**FIG. 7.** Effect of $3 \times 10^{-2}$ M phosphate concentration on bacterial influent and effluent concentration and breakthrough data.

![Figure 8](image8.png)

**FIG. 8.** Effect of $10^{-2}$ M phosphate concentration on a representative bacterial influent and effluent concentration and breakthrough data. Average data with error bars are shown in Fig. 6.

![Figure 9](image9.png)

**FIG. 9.** Effect of $10^{-3}$ M phosphate concentration on representative bacterial influent and effluent concentration and breakthrough data. Average data with error bars are shown in Fig. 6.
ria1 breakthrough curves shown in Fig. 6 is discussed elsewhere (3). These equations use two bacterial attachment parameters: \( \alpha \), which is described in the introduction, and \( n \), which is a bed ripening index. Bed ripening is an observed increase in bacteria attachment rate with time of filtration and is attributed to previously attached bacteria acting as additional attachment sites. These model parameters cannot be theoretically predicted with sufficient accuracy, but their values can be obtained by fitting the numerical simulations to the experimental breakthrough data. Accurate measurement of breakthrough data is therefore important.

Values of \( \alpha \) and \( n \) (Table 1) were obtained by fitting the model to the breakthrough data shown in Fig. 7 to 9. Two sets of parameter values were obtained, one by fitting the model to the breakthrough data obtained by the improved method and the other by fitting the model to the uncorrected data. The improved method has corrected the value of \( \alpha \) at the highest salt concentration, \( 3 \times 10^{-2} \) M, by 17%. This difference is less for the lower salt concentrations. The value of \( n \) has been corrected by 20% at \( 3 \times 10^{-2} \) M salt concentration, and this difference also narrows for lower salt concentrations. Thus, use of the traditional method could yield model parameter values that are in error by as much as 20%. The expected uncertainty in the experimental data obtained using the improved method is less than \( \pm 6\% \), as seen from Fig. 6. This uncertainty is smaller than the 20% variance between the results in Table 1, indicating that the improved method leads to an improvement in the calculated transport and attachment parameters.

![Fig. 10. Effect of \( 10^{-7} \) M phosphate concentration on bacterial influent and effluent concentration and breakthrough data. Average data with error bars are shown in Fig. 6.](image)

Analysis of the decrease in the cell absorbance at column inlet. To further investigate the reasons for the decrease in cell suspension absorbance at the column inlet, it was necessary to study the effects of stirring and/or long-time contact with the phosphate salts on bacterial absorbance. Contact with the phosphate salts might cause the cells to shrink in size if the cells adjust to the higher salt concentrations by excreting some liquids (19).

Microscopic counts (phase contrast, magnification of \( \times 400 \)) were conducted to look for evidence of cell lysis, but no evidence for this was found. Shrinkage was not detectable at a magnification of \( \times 400 \), though this cannot be ruled out because of the limitations of our microscope. Microscopic observations did not show any cell aggregates either. Stirring of the bacterial suspension as a cause of the decrease in cell absorbance was ruled out because the \( C_n \) values were constant for low salt concentrations as shown in Fig. 9 to 11. Stirring in the presence of salts was investigated by using the criterion for cell aggregation (1).

In equation 5,

\[
r_p = \left( \frac{3}{4 \pi C_o} \right)^{1/3} \frac{1}{a_p}
\]

\( r_p \) is dimensionless average interparticle spacing, \( C_o \) is the number concentration of the particles in bulk, and \( a_p \) is the particle radius. The relationship between the \( A_{\text{bio}} \) of the bacterial suspension and the bacterial concentration, \( C \) (cells per milliliter), is obtained using microscopic direct counts (15) as follows:

\[
C = 1.03 \times 10^9 A_{\text{bio}}.
\]

For systems in which \( r_p \geq \left( \frac{1}{2} + \frac{1}{\kappa a_p} \right) \), the suspension is considered dilute enough to neglect interparticle interactions in bulk, where \( \kappa \) is the Debye-Hückel parameter. For our system, \( r_p = 87 \) from equation 5, and \( \kappa = 6.4 \times 10^9 \) m\(^{-1}\) at 0.1 M phosphate salts, and \( a_p = 10^{-6} \) m, which makes the term \( \left[ \frac{1}{2} + \frac{1}{\kappa a_p} \right] \) close to unity. This indicates that the suspension was dilute enough with salts and cells to avoid cell aggregation in the bulk.

One possible reason for the observed decrease in the column inlet cell suspension absorbance readings is a change in the cell dimensions due to changes in salt concentration and osmotic pressure. Koch (11) had attributed the optical changes in \textit{Escherichia coli} due to osmotic change to shrinkage. Another possibility is a change in the refractive index of the bacterial cell surface over time. For the spectrophotometric method to be an even more useful cell detection technique, more research is needed to investigate the physiological response of microorganisms in the subsurface to high salt concentrations.

Conclusions. We have described an improved method to study the effects of ionic strength on bacterial transport in a porous medium of a clean-quartz coarse sand. The method modifies the traditional packed-bed technique by minimizing the time of contact between salts and bacterial suspension before entry into the porous media. Further, continuous monitoring of inlet bacterial suspension is shown to be essential in

![Fig. 11. Representative bacterial influent and effluent concentration and breakthrough data when bacteria are suspended in deionized water. Average data with error bars are shown in Fig. 6.](image)

<table>
<thead>
<tr>
<th>Salt concn (M)</th>
<th>( \alpha )</th>
<th>( n )</th>
<th>( \alpha ) (uncorrected)</th>
<th>( n ) (uncorrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 3 \times 10^{-2} )</td>
<td>0.206</td>
<td>0.728</td>
<td>0.176</td>
<td>0.606</td>
</tr>
<tr>
<td>( 10^{-2} )</td>
<td>0.163</td>
<td>0.710</td>
<td>0.162</td>
<td>0.639</td>
</tr>
<tr>
<td>( 10^{-3} )</td>
<td>0.094</td>
<td>0.733</td>
<td>0.103</td>
<td>0.766</td>
</tr>
</tbody>
</table>

\* Corresponding breakthrough data are shown in Fig. 7 to 9.
order to account for a small transient in the inlet cell absorbance readings which occurs at higher salt concentrations. The method is fast and simple, and it leads to more accurate breakthrough data. This method can also be used to study the effect of other solution properties such as pH or surface-modifying agents, where it is desired to minimize the impact of these parameters on bacterial concentration monitoring using light absorbance during long-term bacterial transport experiments.

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