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Effect of Soil-Water Separation Technique on the Estimation of Bacterial Adsorption onto Soil

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ABSTRACT

Adsorption is one of the most significant processes in dictating the fate of bacteria and viruses in soils. Knowledge of adsorption characteristics is, therefore, essential for the prediction of the migration path of such microorganisms. These characteristics include the adsorption capacity, soil-microorganism partitioning coefficient, the state of equilibrium and the applicable isotherm. The techniques used for the determination of those characteristics may have an effect on the outcome in terms of the parameters involved. In this study, laboratory batch experiments using a tracer bacterium, nalidixic acid resistant *Escherichia coli (E.coli* NAR), were carried out to study the effect of three soil-water separation techniques on determining the adsorption characteristics of bacteria onto soil. The techniques used are: centrifugation, sedimentation and filtration. The procedure involved measurements of biotracer concentration in the soil solution before and after the soil-water separation. The filtration technique was found to have the most pronounced effect on the adsorption of *E.coli* NAR onto soil. On the other hand, separating soil and water using the sedimentation technique gave the smallest amount of biotracer adsorption.

INTRODUCTION

Adsorption is perhaps the most significant abiotic process in the subsurface environment for removing bacteria from water percolating through the soil (Canter, 1987; Venhuizen, 1995). In order to adhere, the bacteria produce proteinaceous extracellular adhesive structures (appendages) that bridge repulsive electrostatic forces on submerged substrata (Quintero et al., 1998). This process may take place quite rapidly. Reported equilibrium times for bacterial and viral adsorption were between 30 minutes and 24 hours (Abu Ashour, 1994; Maurice *et al*., 2004).

The physical and chemical characteristics of soil and the type and size of bacteria have a significant effect on the extent and strength of bacterial adsorption onto soil. Attempts to predict the degree of adsorption as a function of either soil properties or characteristics of bacteria have not been very successful. Therefore, it is necessary to conduct laboratory experiments to determine the adsorption characteristics of a particular strain and soil (Corapcioglu and Kim, 1995). Such experiments usually include batch reactors and/ or soil columns. In these experiments, measurement of the extent of bacterial adsorption involves a liquid-solid separation technique such as sedimentation, filtration and centrifugation. The results may differ depending on the technique used.

Solids can be separated from the liquid phase mechanically by filtration, centrifugation or gravitational sedimentation. Separation by gravitational sedimentation

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is generally an effective technique for removal of unstable and destabilized suspended solids from water and waste (Weber *et al*., 1991). Centrifugation is most often used for laboratory-scale harvesting of cells (Gerhardt *et al*., 1994).

Filtration is one of the most common applications in chemical and biological laboratories. The separation of solids from liquid is accomplished by forcing the fluid through a porous membrane. The solid particles are trapped within the pores of the membrane and form a layer on the surface of the membrane (Fonst et al., 1980). Once this layer has formed, its surface acts as a filter medium (Perry *et al*., 1973). Filtration is commonly used in wastewater treatment for the removal of the biological flocs from effluent after secondary treatment (Weber *et al*., 1991).

This paper aims at determining the adsorption characteristics of bacteria onto soil using three techniques of soil-water separation; namely, sedimentation, filtration and centrifugation. The effect of using these techniques on the evaluation of the adsorption characteristics of bacteria onto soil will be investigated.

MATERIALS AND METHODS

Numerous laboratory experiments were carried out using batch experiments involving the adsorption of bacteria onto solids. The specific objectives of this paper are 1) to test the survival of the biotracer in soil and water environments and 2) to determine the effect of the soil-water separation technique on the adsorption characteristics of bacteria onto soil.

Materials

The biotracer chosen for this study is a strain of *Escherichia coli*, which is resistant to nalidixic acid (*E.coli* NAR). This biotracer is non-pathogenic and has proven to be a good indicator of other naturally occurring strains of *E.coli* which can originate from sources such as wastewater of warm-blooded animals because it behaves similarly (Joy *et al*., 1992).

The soil samples were analyzed to determine the soil texture and organic matter content. Using boyoucos hydrometer 152H, the soil texture was classified as loam: 33% sand, 42% silt and 25% clay. The organic content was determined using the Walky Black method to be 0.5% on mass basis.

Batch adsorption experiments were conducted at room temperature in 250 mL sterile Erlenmeyer flasks using 100 mL of solution. The solution contained varying concentrations of biotracer, distilled water and 20 g of soil, in addition to a blank sample used as a control. The pH of the soil solution was measured to be 9. The water and the glassware were sterilized by autoclaving, and asepsis was maintained during all batch experiments.

Methods

The techniques used for soil handling, inoculum preparation and series of batch experiments conducted to determine potential accuracy, survival of *E.coli* NAR, time to equilibrium and adsorption characteristics of biotracer onto soil using three different separation techniques are described below.

Soil Preparation and Storage

The soil was collected from the top 20 cm. First, it was crushed to break up large clumps without changing the soil texture. After air-drying for 3 days, the soil was passed through a 2-mm sieve. Some of the large clumps which retained by the sieve were crushed by hand and sieved again. Finally, the sieved soil was mixed thoroughly and divided into smaller batches, which were kept in sealed plastic bags and stored at room temperature.

The soil water content of the samples in the sealed bags was measured by drying the samples at 103-105 ^{o}C for 24 hr. The average soil water content was 8.2%.

Inoculum Preparation and Storage

A lyophilized culture of *E.coli* NAR was used as a starter culture. A loopfull of the lyophilized culture which was maintained at 4 ^{o}C was inoculated to 100 mL Erlenmeyer flask containing 25 mL Tryptic Soy Broth (TSB; Difco, Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 100 µg/mL nalidixic acid,

and incubated at 28 ºC for 17 to 19 hours The culture prepared was stored at 4 ºC and subcultured at weekly intervals.

At the beginning of each experiment, *E.coli* NAR was increased by adding 0.1 mL of prepared inoculum to 100 mL Erlenmeyer flask containing 25-50 mL TSB supplemented with 100 µg/mL nalidixic acid, depending on the size of inoculum required for each experiment, and incubated at room temperature. The density of cells in the prepared inoculum was between 10^9 and 10^{10} Colony Forming Units (CFU)/ mL.

Biotracer Concentration

There are several methods used to quantify viable cells in bacterial culture, water and soil solution, such as direct microscopic counts, chemical methods for estimating cell mass or cellular constituents, turbidimetric measurements for increases in cell mass and the serial dilution-agar plate method (spread plate technique) (Cappucciuo and Sherman, 1996). The latter method was adopted in this research to determine the biotracer concentration.

In this method, which was used in all experiments, only viable cells are counted (Cappucciuo and Sherman, 1996). Soil suspension samples were diluted by 1:10 into sterile distilled water to a certain order of concentration. Afterwards, a known size, usually 0.1 mL, was spread on TSB-NA agar plate (90×15 mm Petri plate) supplemented with100 µg/mL nalidixic-acid, and incubated at 44 ºC for 24 hours. The colonies were counted using either the Quebec colony counter or an electronic counter. Plates suitable for counting must not contain less than 30 or more than 300 colonies (Cappucciuo and Sherman, 1996). The total count of the suspension (the concentration) was obtained by dividing the number of cells per plate by the dilution ratio. For better accuracy, dilutions were plated in triplicates and the arithmetic means of the three appropriate plate counts were computed.

Survival of *E.coli* NAR in Soil and Distilled Water

To test the survival of *E.coli* NAR in soil and distilled water, two 250-mL sterile Erlenmeyer flasks were used, one containing soil and the other only distilled water. Distilled water was added to the first flask containing 20 g of dry soil and 1mL of inoculum to get a total volume of 100 mL. The soil in the flask was mixed thoroughly. Samples of 1 mL were taken at different times over a period of 7 days and analyzed for bacterial levels.

The second flask contained 1 mL of inoculum and 99 mL of distilled water. Samples of 1 mL were taken at time intervals for 7 days and analyzed for bacterial content. During the 7 days, the two flasks were kept at room temperature and covered loosely to allow the passage of air. Samples were serially diluted, spread on TSB-NA agar plate, supplemented with 100µg/mL nalidixic acid, incubated at 44 ºC for 24 hr and then analyzed for the concentration of the biotracer.

Adsorption Characteristics of *E.coli* NAR onto Soil

A series of laboratory batch experiments was designed to determine adsorption characteristics of the biotracer onto soil using centrifugation, sedimentation and filtration separation techniques.

Biotracer Separation by Centrifugation Technique

The centrifugation technique used in this study is the differential centrifugation widely used in cell fractionation, in which samples are centrifuged for a given time at a given speed, after which the supernatant is decanted. This technique is useful for separating of particles with distinctly different settling velocities (Gerhardt *et al*., 1994).

In order to test the effect of changing inoculum concentration on the extent of adsorption, six 250 mL Erlenmeyer flasks, each containing 20 g of soil, were each inoculated with 10 mL of different inoculum concentration ranging from 10^9 to 10^4 CFU/mL. Distilled water was added to each flask to get a total volume of 100 mL. The flasks were shaken at 200 rpm on an orbital incubator shaker for 24 hours, at room temperature to ensure equilibrium distribution of *E.coli* NAR in the soil-water system. After shaking, a 1 mL sample was taken from the middle of each flask. The biotracer cells contained in these samples represent the cells'

concentration (C_m) in the soil solution regardless of whether they were adsorbed to the soil particles or not. Then the contents of the flasks were mixed thoroughly again, and aseptically 6 mL samples were transferred to centrifuge tubes and centrifuged at 2000 rpm for 1 min. Samples of 1-mL were then withdrawn from the supernatant. Biotracer cells' concentration (C_u) present in these samples was the non-adsorbed cells plus those attached to the soil particles that remained in suspension after centrifugation.

Another set of six 250 mL Erlenmeyer flasks, containing 10 mL of different inoculum concentration ranging from10^4 to 10^9 CFU/mL, plus 90 mL of distilled water and no soil, were used as control samples and were treated exactly in the same way. After shaking, a 1-mL sample was taken from the middle of each flask. The control flasks were exactly treated in the same way and the samples were taken in the same manner. All samples collected were serially diluted into sterile distilled water, plated on TSB-NA agar and incubated at 44 ^oC for 24 hours before bacterial enumeration.

Biotracer Separation by the Sedimentation Technique

The same procedure previously described was followed here as well, except that after withdrawing the samples from the middle of each flask, the flask contents were mixed vigorously and soil suspensions were poured into sterile 100 mL graduated cylinders, which were allowed to settle for 60 minutes. After settling, samples were taken from the top 2 cm using sterile tips to determine the non-adsorbed cells and those attached to finer soil particles that remained suspended in the 2 cm. The control flasks were exactly treated in the same way. All samples collected were serially diluted, plated on TSB-NA agar and incubated at 44 ºC for 24 hours to determine bacterial level.

Biotracer Separation by the Filtration Technique

The last method of separation is filtration. To determine the non-adsorbed cells, first a 1-mL sample taken from the middle of each flask was filtered using a microporous cellulose acetate filter, 30mm in diameter. Before filtering, the sample was diluted twice into sterile distilled water to reduce loading on filters and prevent filter clogging that may form a surface cake or mat that provides extra retention. A 4-mL volume of the second dilution was filtered using a sterile syringe through a membrane filter with precision-sized holes of 3 µm. This pore size permitted passage of only bacteria, water and very fine soil. The same treatment was performed in the case of the six control flasks, except that there was no need for dilution before filtering. After that, a 1-mL sample of the filtrate was serially diluted, plated on TSB-NA agar and incubated at 44 ºC before bacterial enumeration.

The fraction of soil remaining in suspension with bacteria was determined after separation in the supernatant or filtrate was determined. The soil content was measured by the gravimetric method in 1-mL samples taken from the middle of each flask and in 1-mL samples withdrawn after centrifugation, sedimentation or filtration. The corresponding values of soil fraction remaining in suspension after filtration, centrifugation and sedimentation were 0.08, 0.09 and 0.08, respectively. In our analysis, all biotracer cells adsorbed on this fraction of soil were treated as not adsorbed.

RESULTS AND DISCUSSION

The extent of bacterial adsorption in soil is affected by mechanical filtration (Abu-Ashour *et al.*, 1994) and interstitial velocity (Abu-Ashour and Abu-Zreig, 2005). In order to study the adsorption of bacteria onto soil without the effects of these factors, it was necessary to develop a suitable technique for assessing the extent to which bacteria adhere to soil. Three soil-water separation techniques were used to study the adsorption phenomena: centrifugation, sedimentation and filtration.

Good biotracer survival in soil and distilled water is essential for the success of the experimental procedure adopted. The results shown in Figures (1) and (2) indicate that during the 7-day test period, the biotracer sustained its concentration within 5% of the initial concentration in

both soil and distilled water. The good survival of the biotracer may be attributed to the low concentration of predators competing for the available nutrients. Hence, the decay of the biotracer in distilled water has no effect on the soil-batch experiments.

The ratio of biotracer concentration in the supernatant or filtrate (C_u) and the biotracer concentration in the soil suspension (C_m) were drawn versus initial inoculum concentration for all separation techniques. As mentioned earlier, (C_u) represents the non-adsorbed. Hence, a ratio (C_u/C_m) closer to 1 indicates weaker biotracer adsorption by soil. As shown in Figure (3), the filtration technique was found to have the most pronounced effect on the adsorption of *E.coli* NAR onto soil. On the other hand, the sedimentation technique gave the smallest amount of biotracer adsorption. The initial inoculum concentration did not seem to have an effect on the results.

The ratio (C_u/C_m) in control flasks containing the biotracer in distilled water is a measure of the effect of the buoyancy of the biotracer cells. As shown in Figure (4), (C_u/C_m) values were close to 1 for all separation techniques indicating that the biotracer cells remained buoyant after separation. This is due to the fact that the density of biotracer almost equals the density of water.

It is clear from the previous discussion that adsorption characteristics are affected by the type of the separation technique used. This warrants the recommendation that the method of estimating adsorption parameters should be reported.

CONCLUSIONS

The survival of *E.coli* NAR in soil and distilled water were excellent during the test period. The biotracer cells were not affected by the separation technique due to their size and density. Among the three applied separation techniques tested, the highest estimate of adsorption was using the filtration technique, while the lowest was for sedimentation. In light of the results obtained in this study, it is recommended that the method of estimating adsorption parameters should be reported.

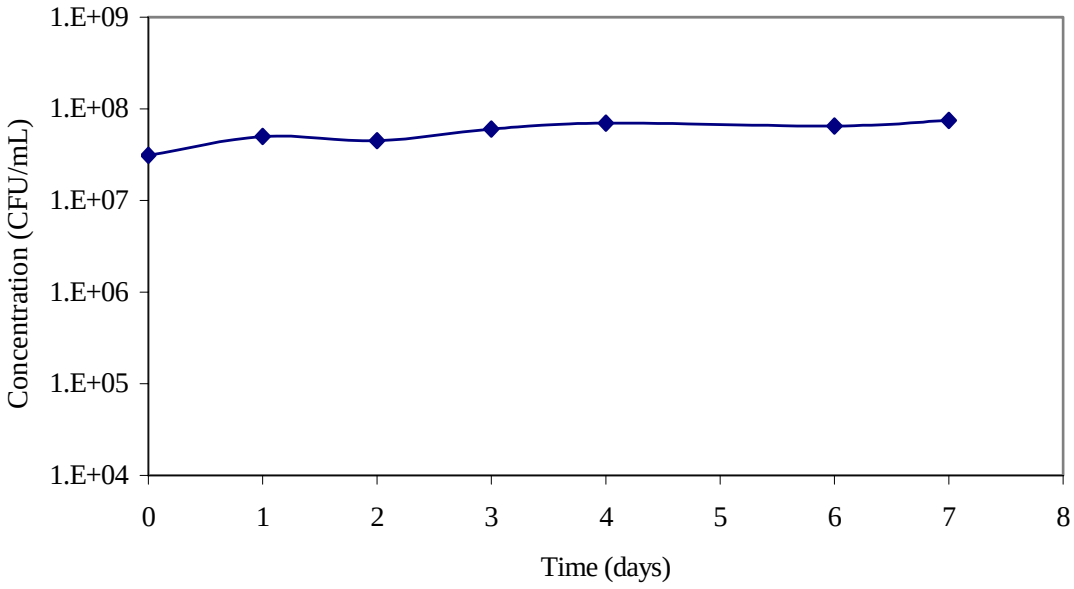

Figure 1: Survival of *E. coli* NAR in distilled water.

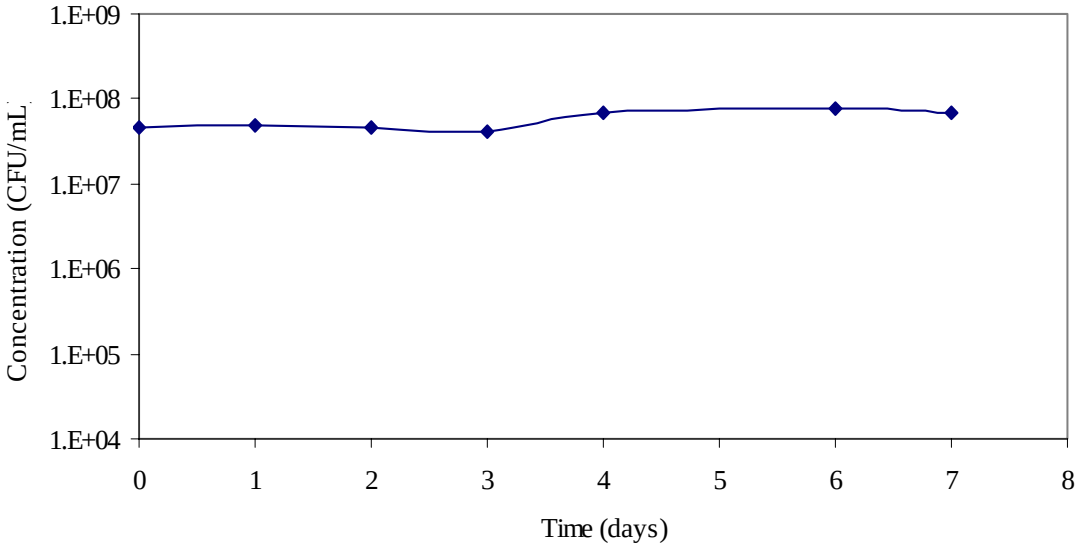

Figure 2: Survival of *E. coli* NAR in soil.

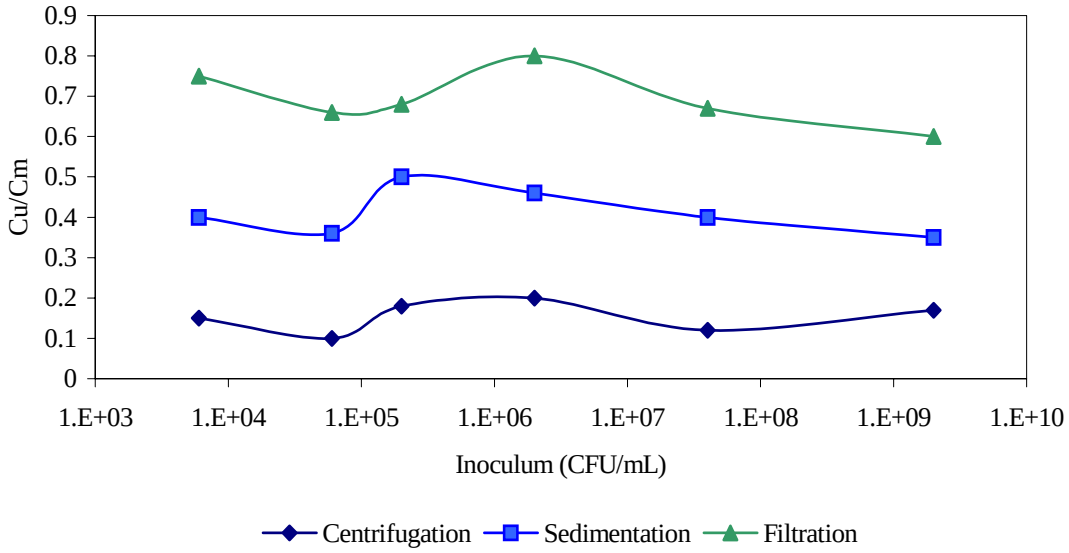

Figure 3: C_u/C_m versus initial inoculum concentration for all separation techniques.

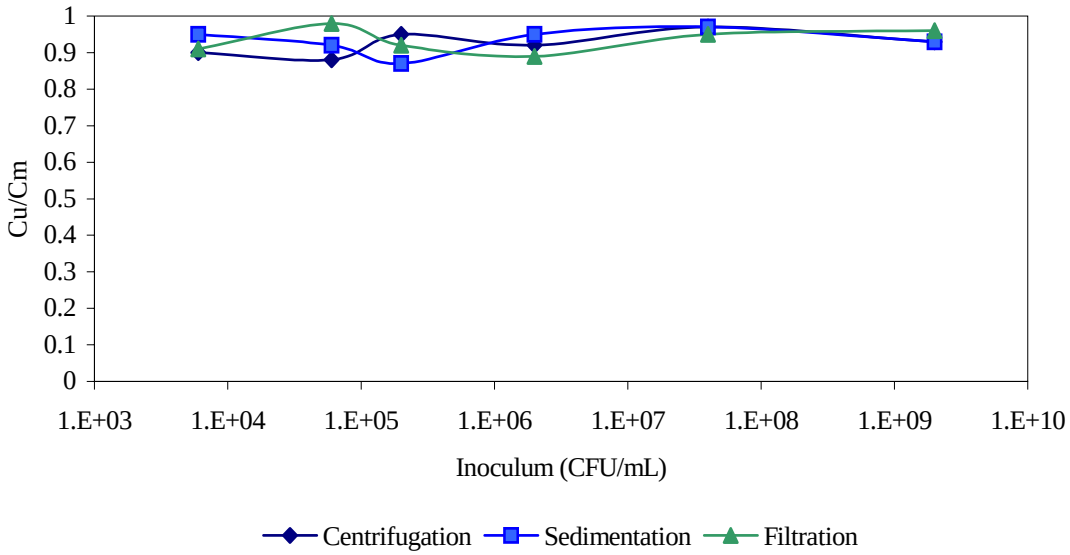

Figure 4: C_u/C_m versus initial inoculum concentration in control flasks for all separation techniques.

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