Transport of antibiotic-resistant bacteria and resistance-carrying plasmids through porous media

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Abstract Microbial antibiotic resistance has emerged as not only a major medical problem but also as an environmental engineering challenge, with antibiotic resistance genetic elements as environmental pollutants. The transport characteristics of a tetracycline-resistant bacterium (*B. cepacia*) and plasmids carrying tetracycline-resistance genes were investigated using flow-through columns packed with porous media. Higher influent cell concentrations (1.1×10^8 CFU mL^{-1}) resulted in higher breakthrough ($C/C_0 = 0.596 \pm 0.055$) than a solution with lower cell concentration (2.0×10^6 CFU mL^{-1}, $C/C_0 = 0.461 \pm 0.037$). This decreased extent of filtration suggests fast initial cell deposition and strong subsequent blocking of binding sites, resulting in less-hindered microbial transport through the sandy medium. The addition of a bromide tracer (NaBr) prior to the plasmid solution resulted in DNA retardation and increased filtration in a zirconia-silica bead matrix. Apparently, Na^+ binding to the beads decreased electrostatic repulsion between the negatively charged DNA and zirconia-silica surface. In contrast, plasmid breakthrough preceded that of the tracer when the plasmids were added first, possibly due to size exclusion chromatography coupled with stronger electrostatic repulsion. This implies that efforts to characterize the dynamics of resistance vector propagation in aquifers should consider the effect of groundwater chemistry and the surface characteristics of the porous media on vector transport.

Keywords Antibiotic resistance; plasmid; RTQ-PCR

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Introduction

Microbial antibiotic resistance — often generated by the intensive use of antimicrobial agents in animal agriculture (Mellon *et al.*, 2001) — is receiving increasing attention in light of the increasing incidence of human bacterial infections resistant to antibiotic treatments (Heseltine, 2000). This phenomenon has sparked and reinforced research in areas such as microbial ecology, gene transfer, and use and fate of antibiotics in agricultural settings (Furushita *et al.*, 2003; Stanton and Humphrey, 2003). There has been success in isolating antibiotic resistant pathogens (Guillaume *et al.*, 2000), describing the resistance mechanisms (Roberts, 1996), and developing molecular techniques for the detection of genes responsible for antibiotic resistance (Barbosa *et al.*, 1999). As a result, many of the facets of the resistance problem have been elucidated. However, numerous questions remain about the dynamics of resistance propagation in natural systems, one of which is the motivation for this study: how do environmental factors influence the transport of resistance vectors through soils and aquifers?

Microbial transport in soil and other matrices has been widely investigated (Harvey and Garabedian, 1991; Barton and Ford, 1995; Rogers and Logan, 2000). Factors known to affect the migration and filtration of bacteria in porous media include, but are not limited to, carrier solution ionic strength (Martin *et al.*, 1992), electrophoretic mobility and cell hydrophobocity (Gross and Logan, 1995), and shape and size of the porous matrix material (Brown *et al.*, 2002). However, the effect of groundwater chemistry on microbial

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transport has not been fully elucidated, and the behavior of (free or plasmid) DNA that codes for antibiotic resistance in such setting is relatively unknown.

Free-DNA (released by dead cells) can bind to soil or aquifer material and retain transformational capabilities (Lorenz and Wackernagel, 1987). This suggests the possibility that indigenous microorganisms could acquire antibiotic resistance not only through conjugation, but also by direct uptake, i.e., transformation, of resistance genes released by enteric bacteria from animal feeding lots. Thus, sorbed DNA elements represent a potential additional 'reservoir' for the amplification of antibiotic resistance in soil microorganisms. If antibiotic resistance is considered as an environmental engineering problem, with the genetic elements considered as target pollutants (Rysz and Alvarez, 2004), this additional pool of resistance genes poses a remediation challenges for possible 'clean-up' efforts. Thus, understanding the transport, sorption and degradation behavior of free-DNA elements in porous media, along with the migration of host microorganisms through such matrices, is important for risk assessment and control.

The objective of this work was to study the transport of both free-DNA elements (antibiotic resistance plasmid) and bacteria though porous media using flow through columns. Variables such as the gene vector concentration and the effect of tracer studies were also considered to contribute to our understanding of factors affecting the dynamics of antibiotic resistance propagation.

Methods

General approach

Saturated flow-through columns were used to investigate transport characteristics of bacteria (*Burkholderia cepacia*) containing antibiotic resistance genes and free-DNA elements in porous media. Flow-through glass columns packed with sand or beads were used to simulate the porous matrix, and injected separately with radio-labelled bacteria and plasmid solutions.

Bacterial transport

Bacterial strain preparation. A tetracycline resistant *Burkholderia cepacia* strain carrying a tetC gene (efflux pump resistance mechanism) was isolated and characterized as described elsewhere (Rysz and Alvarez, 2004). Cells were grown on tryptic soy broth amended with tetracycline (50 mg/L) and prepared for transport experiments using the MARK (microbe and radiolabel kinesis) method (Gross *et al.*, 1995). Briefly, cells were incubated overnight in 100 mL MSB solution amended with 75 μL L[3,4,5-^{3}H(N)]-leucine (Perkin Elmer Sciences, 173 Ci mmol^{-1}, 1 mCi ml^{-1}). The cells were centrifuged (13,000 g for 10 minutes) the pellet was resuspended in 100 mL of fresh MSB, pelleted again and resuspended in fresh MSB solution to assure all detectable radioactive signal was due to ^{3}H-luecine incorporated into the bacteria, rather than to background in the original growth medium. The final resuspension MSB volume was adjusted to obtain microbial concentrations of 2.0×10^{6} CFU mL^{-1}, and 1.1×10^{8} CFU mL^{-1}, measured at OD_{600}, with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences). The higher concentration is representative of raw wastewater.

Column preparation and operation. Glass columns (15 cm long, 1 cm ID, Kontes Glass, Co.) were fitted with threaded flow adapters and packed with sand ($f_{oc} < 0.1\%$). The influent solution was delivered via a Harvard Apparatus 22 syringe pump (Harvard Bioscience, Inc) equipped with a 100 mL gas-tight syringe (SGE International, Pty. Ltd.). A bromide tracer test was conducted prior to the injection of the bacterial solution. The effluent of the column was collected and analyzed for the concentrations of the

conservative tracer and ^{3}H-leucine-labeled bacteria. The bromide concentration (NaBr influent 600 mg L^{-1}) was measured with the Accumet® detector (Fisher Scientific) equipped with a bromide combination electrode (Cole Parmer Instrument Co.). Aliquots of the effluent bacterial solution (1 mL) were added to 10 mL scintillation cocktail (ScintiSafe Plus, Fisher Scientific) and analyzed with an LS 6500 Scintillation Counter (Beckman Coulter, Inc.). Effluent concentrations of bromide and bacteria were normalized to the respective influent concentrations.

The one-dimensional (1D) advection–dispersion model (Equation 1) (Domenico and Schwartz, 1998) was used to determine column hydraulic parameters (retardation factor (R_f) = 1, effective porosity (η_e) = 0.4, and the dispersion coefficient ($D = 5.5\,cm^2\,hr^{-1}$), based on measured values of C (effluent bromide concentration), C_o (influent bromide concentration) x (column length), t (elapsed time), Q (flow rate), and A (column cross sectional area).

$$C = \left(\frac{C_o}{2}\right)\mathrm{erfc}\left[\frac{\left(R_f x - \left(\frac{Q}{A\eta_e}\right)t\right)}{2\sqrt{DR_f t}}\right] + \exp\left[\frac{\left(\frac{Q}{A\eta_e}\right)x}{D}\right]\mathrm{erfc}\left[\frac{\left\{R_f x + \left(\frac{Q}{A\eta_e}\right)t\right\}}{2\sqrt{DR_f t}}\right] \quad (1)$$

DNA transport

DNA preparation. Plasmid DNA used in the transport experiments was harvested from overnight cultures of *E. coli* c600 harboring the pSC101 vector containing the tetC determinant antibiotic resistance gene (ATCC#37032), with the UltraClean™ Maxi Plasmid Prep Kit (MoBio Laboratories) according to manufacturer's extraction protocol. The concentration of the extracted DNA was determined with the Ultrospec 2100 pro spectrophotometer at 260 nm.

Column preparation and operation. Similar glass columns were packed with 0.5 mm zirconia-silica beads (Biospec Products, Inc.). Influent solutions were delivered via a Harvard Apparatus 22 syringe pump (Harvard Bioscience, Inc) equipped with a 100 mL glass syringe (SGE International, Pty. Ltd.).

Two regimens were used to study free-DNA transport in which each solution (sterile DNAse-free water with plasmid and bromide tracer) was added separately to the column. In regimen 1, the column was washed with sterile DNAse-free water, followed by the addition of the bromide tracer, and subsequently the plasmid DNA solution. In the second treatment, the column was washed with sterile DNAse-free water, followed by the addition of the DNA solution, and finally the bromide tracer. In each scenario column effluent was collected and analyzed for the concentrations of bromide (NaBr influent 600 mg L^{-1}) with the combination electrode. Aliquots of the effluent samples were analyzed for the absolute concentration of the tetC gene by RTQ-PCR (real time quantitative PCR) method. The concentration of the influent (initial) DNA solution was determined spectrophotometrically ($C_o = 0.2\,\mu g\,mL^{-1}$). Effluent concentrations of bromide and free-DNA were normalized to the respective influent concentrations.

Sample analysis. Samples of the column effluent were analyzed for the presence of the tetC gene by RTQ-PCR with the 7500 Real Time PCR System (Applied Biosystems). Reaction mixtures (25 μL) containing 1X SYBR® Green Master Mix (Applied Biosystems), 2 μL aliquots of the effluent samples, forward (GCCTATATCGCCGACATCAC) and reverse (GTAGGTTGAGGCCGTTGAGC) primers at 500 nM reaction concentration (Stanton and Humphrey, 2003) were amplified as follows: 1 minutes at 50 °C, 10 minutes at 95 °C, 40 cycles of denaturation for 15 seconds at 95 °C, annealing for 1 minute at 60 °C,

followed by the dissociation stage cycle (15 seconds at 95 °C, 1 minute at 60 °C, 15 second at 95 °C).

Aliquots of selected column effluent samples were also analyzed by conventional PCR. Reaction mixtures (50 μL)containing 5 μL of effluent DNA sample, 0.25 μM forward and reverse tetC primers (Stanton and Humphrey, 2003), and 25 μL of the HotStarTaq® Master Mix Kit (Qiagen Inc., CA. USA) were amplified as described previously (Stanton and Humphrey, 2003). PCR products were separated on a 1.2% agarose gel, and visualized with an UV transilluminator (UVP Inc., CA, USA)

Results and discussion

In the current study we focused on the transport characteristics of antibiotic resistant bacteria and the DNA elements responsible for such resistance. Flow-through columns packed with various porous matrices were well suited for the experiments as they may represent the characteristics of typical groundwater movement and infiltration events that occur at sites 'contaminated' with such resistance vectors.

Antibiotic resistant bacterial transport

Migration characteristics of the antibiotic resistant bacterium (*B. cepacia*) are presented in Figure 1. Both high (2.0×10^6 CFU mL^{-1}) and low (1.1×10^8 CFU mL^{-1}) influent bacterial concentrations experienced significant retention, i.e., filtration, within the column, as indicated by steady-state effluent concentrations significantly lower than the influent concentration. The column with the high influent bacterial concentration resulted in a higher normalized breakthrough level ($C/C_0 = 0.596 \pm 0.055$) compared to the column with the lower influent microbial concentration ($C/C_0 = 0.461 \pm 0.037$). The (statistically significant) lower extent of filtration observed for the higher influent bacterial concentration is counterintuitive to the predictions of traditional colloidal filtration theory (Yao *et al.*, 1971). This observation may be explained by considering the clean bed collision efficiency parameter (α_o), which describes the initial cell deposition, and the blocking factor (B), which accounts for coverage of the solid surface by attached cells and indicates the extent of cell-cell interactions (Rijnaarts *et al.*, 1996a). The correlation between α_o and B creates bacterium/collector regions in which distinct deposition and blocking patterns influence potential bacterial transport. As defined, region II is characterized by

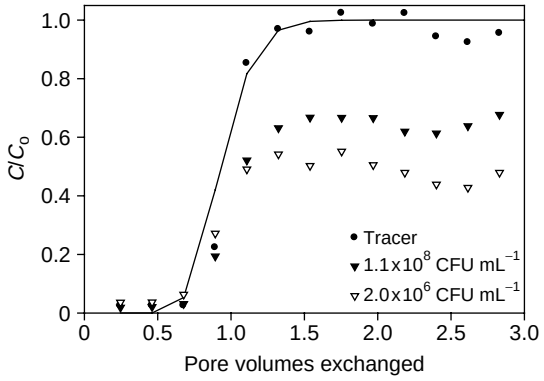

Figure 1 Bacterial transport and filtration through saturated sand columns. Solid line represents tracer model (Equation 1). The effect of influent cell concentration on breakthrough patterns suggest high initial deposition behavior (high α_o) coupled with strong blocking (high B) and less-hindered microbial transport (at saturation) for the solution with higher influent cell concentration

absence of clogging and an unhindered microbial transport after surface saturation due to the fast initial cell depositions and strong blocking.

Pseudomonas putida mt2 was shown to exhibit region II behavior (Rijnaarts *et al.*, 1996b). We hypothesize that the *B. cepacia* strain (originally known as *Pseudomonas cepacia*; Snell *et al.*, 1972) used in this study possessed similar macromolecular cell coating components to *P. putida mt2* and exhibited a similar region II behavior. Therefore, we postulate that fast initial deposition (characteristic of high α_o values) and strong blocking at saturation (characteristic of high B values) occurred when the higher cell concentration was fed through the column, and that this led to relatively unimpeded cell transport and higher cell breakthrough (Figure 1).

DNA transport

Similar to the bacterial transport experiments, the 1D advection–dispersion equation was used to model the bromide tracer data for the columns used in DNA transport experiments ($\eta_e = 0.5$, $D = 0.5\,cm^2\,hr^{-1}$). regimen 1, tracer addition followed by DNA solution (Figure 2), resulted in overall retardation and increased filtration of the plasmid DNA, while for regimen 2, DNA solution followed by tracer (Figure 3), the tetC gene breakthrough preceded that of the tracer with less filtration. These results suggest that the porous media surface chemistry strongly affects the breakthrough characteristics of mobile DNA elements carrying antibiotic resistance genes.

The retardation and filtration of the plasmid solution in regimen 1 (Figure 2) is probably due to a change in the surface charge of the column packing material upon addition of the tracer solution. We postulate that the presence of sodium cations contained in the tracer solution (NaBr) imparted a positive charge on the negatively charged zirconia-silica beads, forming bead-sodium complexes that increased electrostatic attraction for (and retardation plus filtration of) the negatively-charged DNA molecules. In the absence of sodium ions (regimen 2) the phenomenon of size exclusion chromatography (SEC), coupled with the net negative charge on the packing beads, was responsible for the early elution of the plasmid DNA. SEC involves the preferential exclusion of bacterial cells from smaller, more tortuous pores between column packing material, resulting in a more direct average path of travel and earlier elution times (Harvey *et al.*, 1989). Although bacterial cells are much larger (0.1–2 μm diameter) than the plasmid molecules (size range 3.5–24 nm; Bustamante *et al.*, 1992), the repulsive electrostatic effect exerted by the negatively-charged column beads on the negatively-charged plasmid molecule likely

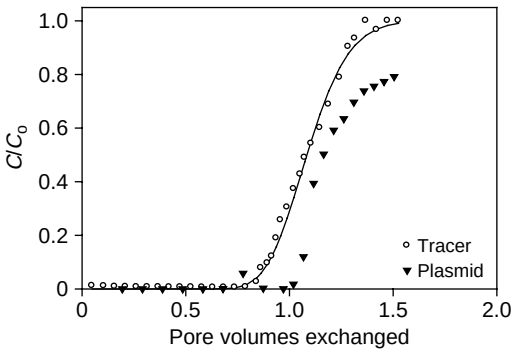

Figure 2 Normalized DNA and bromide concentrations for transport regimen 1 (column packed ⇒ H_2O wash ⇒ tracer ⇒ DNA). Solid line represents tracer model (Equation 1). The observed DNA behavior suggests that addition of the bromide tracer prior to DNA resulted in a change of porous media surface chemistry leading to retardation and decreased filtration (as compared to regimen 2) of plasmid DNA

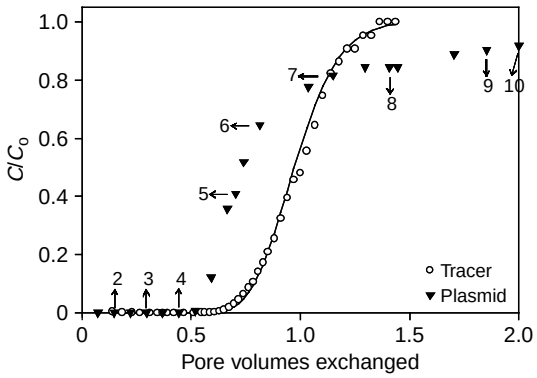

Figure 3 Normalized DNA and bromide concentrations for transport regimen 2 (column packed ⇒ H_2O wash ⇒ DNA ⇒ tracer). Solid line represents tracer model (Equation 1). Earlier DNA elution times (compared to tracer) and decreased DNA filtration (compared to regimen 1) suggests SEC and higher electrostatic repulsion between negatively-charged bead surfaces and DNA. Numbered samples represent aliquots analyzed by PCR (Figure 4)

contributed to the SEC mechanism as suggested by the earlier elution time and lesser degree of filtration of the plasmid DNA in regimen 2 (Figure 3) compared to regimen 1 (Figure 2).

The presence of plasmid DNA in the effluent samples of bead-packed columns (regimen 2) was also determined by standard PCR (Figure 4). Although only some of the column breakthrough samples were analyzed by PCR, the results confirm the increase of plasmid DNA concentration (detected tetC gene) in the column effluent as evidenced by the increasing intensity of the tetC amplicons. Although no densitometry analysis was done on the agarose gel, the relative intensity of the PCR products in Figure 4 (especially lanes 5 and 6), were used to corroborate the results of the transport experiments.

In order to mimic more natural conditions, we attempted to study DNA transport in sand columns, but the RTQ-PCR method did not yield satisfactory results. Apparently, the DNA in the effluent samples of the sand column was not detected due to the sensitive nature of the RTQ-PCR method, which relies on DNA samples free of PCR reaction inhibitors. Substances such as humics, DNases, and free ions that could have washed off the sand matrix during the experiment, most probably prevented amplification of the tetC

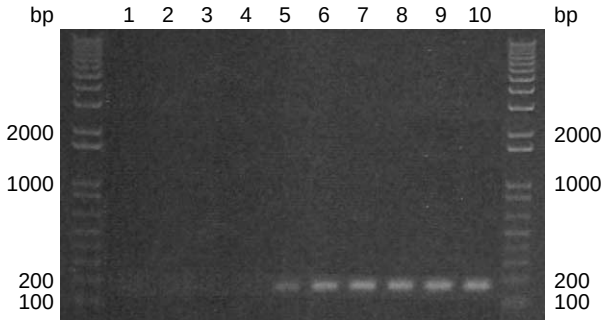

Figure 4 Agarose gel electrophoresis of selected effluent samples (tetC gene) from regimen 2 DNA transport experiment (Figure 3). Lane 1: no DNA control; Lanes 2–10 correspond to PCR amplicons of samples indicated in Figure 3. No product was observed for lanes 2–4 (corresponding to $C/C_0 = 0$, as determined by RTQ-PCR). Sample 5 ($C/C_0 = 0.4098$) produced a detectable amplicon (lesser intensity than samples 6–10, with $C/C_0 = 0.6456$, 0.8171, 0.8451, 0.8889, and 0.9193, respectively) semi-quantitatively indicating the amount of DNA present

gene during the RTQ-PCR analysis. Purification attempts of the effluent DNA by precipitation methods were unsuccessful due to the limited sample volume.

Conclusions

Both groundwater and porous matrix surface chemistry are important factors affecting the transport of bacteria and plasmids through aquifers and soils, and that the sequence in which benchmarking tracers are injected can influence surface chemistry and breakthrough patterns. Thus, the effects of tracer tests on the transport of resistance vectors and other colloids of interest should not be overlooked.

Bacteria and free DNA migration is likely to be attenuated by filtration. Thus, the sorption and persistence of transformation vectors retained in soil could play an important role in serving as potential reservoirs of antibiotic resistance in the environment. This suggests that further research on the roles cell-particle interactions and the influence of environmental factors on vector sorption, filtration, retardation, amplification and attenuation is needed to understand and predict the dynamics of antibiotic resistance propagation.

References

Barbosa, T.M., Scott, K.P. and Flint, H.J. (1999). Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. *Environ. Microbiol.*, **1**(1), 53–64.

Barton, J.W. and Ford, R.M. (1995). Determination of effective transport coefficients for bacterial migration in sand columns. *Appl. Environ. Microbiol.*, **61**(9), 3329–3335.

Brown, D.G., Stencel, J.R. and Jaffe, P.R. (2002). Effects of porous media preparation on bacteria transport through laboratory columns. *Water Res.*, **36**, 105–114.

Bustamante, C., Vesenka, J., Tang, C.L., Rees, W., Guthold, M. and Keller, R. (1992). Circular DNA molecules imaged in air by scanning force microscopy. *Biochemistry*, **31**, 22–26.

Domenico, P. and Schwartz, F.W. (1998). *Physical and Chemical Hydrogeology*, Wiley, New York.

Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., Torri, K., Hasegawa, T. and Ohta, M. (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl. Environ. Microbiol*., **69**(9), 5336–5342.

Gross, M.J., Albinger, O., Jewett, D.G., Logan, B.E., Bales, R.C. and Arnold, R.G. (1995). Measurement of bacterial collision efficiencies in porous media. *Water Res.*, **29**(4), 1151–1158.

Gross, M.J. and Logan, B.E. (1995). Influence of different chemical treatments on transport of *Alcaligenes paradoxus* in porous media. *Appl. Environ. Microbiol*., **61**(5), 1750–1756.

Guillaume, G., Verbrugge, D., Chasseur-Libotte, M., Moens, W. and Collard, J. (2000). PCR typing of tetracycline resistance determinants (Tet A-E) in *Salmonella enterica* serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. *FEMS Microbiology Ecology*, **32**, 77–85.

Harvey, R. and Garabedian, S.P. (1991). Use of colloid filtration theory in modeling movement of bacteria through a contaminated sandy aquifer. *Environ. Sci. Technol.*, **25**(1), 178–185.

Harvey, R.W., George, L.H., Smith, R.L. and LeBlanc, D. (1989). Transport of microspheres and indigenous bacteria through a sandy aquifer: results of natural- and forced-gradient tracer experiments. *Environ. Sci. Technol.*, **23**(1), 51–56.

Heseltine, P. (2000). Has resistance spread to the community? *Clin. Microbiol. Infec.*, **6**(Supp. 2), 11–16.

Lorenz, M.G. and Wackernagel, W. (1987). Adsoprtion of DNA to sand and variable degradation rates of adsorbed DNA. *Appl. Environ. Microbiol*., **53**(12), 2948–2952.

Martin, R.E., Bouwer, E.J. and Hanna, L.M. (1992). Application of clean-bed filtration theory to Bacterial Deposition in Porous Media. *Environ. Sci. Technol.*, **26**(5), 1053–1058.

Mellon, M., Benbrook, C. and Benbrook, K. (2001). *Hogging it: Estimates of Antimicrobial Abuse in Livestock*. Union of Concerted Scientists (UCS) Publications, Cambridge, MA.

Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J. and Zehnder, A.J.B. (1996a). Bacterial deposition in porous media: related to the clean bed collision efficiency and to substratum blocking by attached cells. *Environ. Sci. Technol.*, **30**(10), 2869–2876.

Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J. and Zehnder, A.J.B. (1996b). Bacterial deposition in porous media: effects of cell-coating, substratum hydrophobicity, and electrolyte concentration. *Environ. Sci. Technol.*, **30**(10), 2877–2883.

Roberts, M.C. (1996). Tetracycline resistance determinants: mechanism of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Reviews*, **19**, 1–24.

Rogers, B. and Logan, B.E. (2000). Bacterial transport in NAPL-contaminated porous media. *J. Env. Engng.*, **126**(7), 657–666.

Rysz, M. and Alvarez, P.J.J. (2004). Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments. *Water Res.*, **38**, 3705–3712.

Snell, J.J.S., Hill, L.R., Lapage, S.P. and Curtis, M.A. (1972). Identification of *Pseudomonas cepacia* Burkholder and its synonymy with *Pseudomonas kingii* Jonsson. *Int. J. Systemat. Bacteriol.*, **22**, 127–138.

Stanton, T.B. and Humphrey, S.B. (2003). Isolation of tetracycline-resistant megasphaera elsdenii strains with novel mosaic gene combinations of *tet*(O) and *tet*(W) from swine. *Appl. Environ. Microbiol.*, **69**(7), 3847–3882.

Yao, K.M., Habibian, M.T. and O'Melia, C.R. (1971). Water and waste water filtration: concepts and applications. *Environ. Sci. Technol.*, **5**(11), 1105–1112.