LMECOL2000-01

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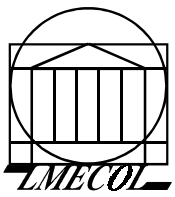

A publication of the Laboratory of Microbial Ecology
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LMECOL Contribution 2000-01, Feb. 2000

Effect of Humic Material on Interactions Between Bacterial Cells and Mineral Surfaces

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The presence of dissolved humate did not affect sorption of bacteria to clean quartz sand at the concentrations studied (up to 1.0 mg liter^{-1}) in batch experiments using 50 ml of artificial ground water (AGW), 25 g sand, and an incubation period of three hours.. A strain effect was observed, with 50% of the added cells of a relatively more hydrophilic, less negatively charged strain (S138) retained across the treatments, but only 2.3% of the added cells of a more hydrophobic, more negatively charged strain (S139) retained. When humate was sorbed to iron-coated quartz sand at two concentrations, but was not present in the AGW, bacterial sorption to iron-coated sand decreased, and sorption decreased with increasing humate concentration in the range examined. An effect of strain was not seen. The presence of a humate coat over a metal oxide coating modifies the sorptive properties with respect to bacterial cells. The results suggest that the organic matter blocks otherwise available sorption sites, and sorption of the bacterial cells to the organic coating does not make up for the loss of the sites associated with the metal oxide.

The phenomenon of bacterial transport through porous media includes the broad categories of advection, dispersion, deposition, entrainment, growth, and death. These processes have been incorporated into various models such as those constructed by Corapcioglu and Haridas (1985) and Peterson and Ward (1989). Fitting models to experimental data (Hornberger, et al. 1992, Peterson and Ward 1989) has shown that consideration of deposition is essential. Processes involved in deposition include straining (wedging between porous media grains), sedimentation (settling due to weight and fluid drag), interception, and sorption. However, many bacteria fall into a size and specific gravity range in which the first three factors are minimized (Herzig, et al. 1970, McDowell-Boyer, et al. 1986). For particles of diameter on the order of 1 μm, Herzig et al. (1970) calculated that surface phenomena (i.e., sorption mechanisms) are most important. Harvey and Garabedian (1991) reached similar conclusions and estimated that sorption was responsible for removal of up to 85% of injected bacteria traveling across 7 m of sandy aquifer.

Factors that affect sorption of bacteria to surfaces include a wide range of characteristics of the bacterium, the substratum, and the surrounding environment (Daniels 1980) However, all of the factors affecting the cell or the substratum, as well as those environmental factors dealing with the composition of the solution or suspension, interact to create two surface properties which have been found to be important by many investigators, although to varying degrees (e.g. see (Mozes, et al. 1987, Van Loosdrecht, et al. 1987a): the charge and hydrophobicity of the surfaces involved. The interaction of attractive and repulsive forces including those established by electrostatic charges and hydrophobicity make up the basis of DLVO theory (Van Loosdrecht, et al. 1990).

Most natural surfaces, including most bacteria, are negatively charged at pH levels normally found in the environment (Fletcher and Marshall 1982, Harden and Harris 1953), so that repulsion would be expected as a result of the interaction of most pairs of natural surfaces and their associated double layers of counterions. Not surprisingly, the charge of the mineral surface and that of the bacterial cell surface also affect bacterial sorption. Fletcher and Loeb (1979), Mozes et al. (1987), Scholl et al. (1990), and Mills et al. (1994) all found that positively charged or neutral surfaces attracted more bacteria than negatively charged surfaces (Fletcher and Loeb (1979) and Mozes et al. (1987) also observed a hydrophobicity effect); the effect of substratum charge was also seen in a transport study by Scholl et al. (1992). Sharma et al. (1985) found that the transport of bacteria through sandpacks was increased if the negative charge of the bacterial surfaces was increased. Van Loosdrecht et al. (1987a) and Stenström (1989) also found that bacterial surface charge had

some effect on sorption, but not to as great an extent as bacterial surface hydrophobicity.

Sorption due to surface charge, hydrophobicity, or any combination of the two would seem a likely phenomenon to be affected by the presence of a coating on the surface of a substratum. Ferric coatings, for instance, were found to greatly enhance bacterial sorption by Mozes et al. (1987), Scholl et al. (1990), and Mills et al. (1994). One of the most ubiquitous of all coatings is that of organic matter. Hunter and Liss (1981) found that, without exception, the suspended particles in river and estuarine waters were negatively and quite uniformly charged. Since the particles themselves varied widely as to composition, they concluded that this was likely due to a coating of organic matter or metal oxide. Immersing positively charged hydrous iron oxide particles in natural water containing organic matter results in a negative charge being imparted to the particles, presumably due to the sorption of organic molecules (Loder and Liss 1985, Tipping 1981, Tipping and Cooke 1982).

The effect of organic matter on bacterial sorption is not well studied. Pringle and Fletcher (1986) found that a variety of macromolecules inhibited attachment of bacteria to polystyrene when the macromolecules were present in the suspension during the attachment period, and a number of the macromolecules also inhibited attachment when the surfaces were preconditioned with them. Scholl and Harvey (1992) observed that retention of bacteria in artificial ground water was greatest in sand which had been leached of organic matter but still retained an iron-oxyhydroxide coating (and was therefore positively charged), as opposed to sand which had both an organic and iron coating or sand which had neither. They also obtained less sorption to sand that was coated both with iron oxyhydroxide and organic matter when organic matter was present in the suspension (but not with iron-coated sand with the organic-matter coating removed, as might be expected for a competition mechanism). Scholl and Harvey (1992) concluded that experiments with better-defined components were necessary. Both Lance and Gerba (1984) and Powelson et al. (1991) found that organic matter in solution (derived from sewage sludge in both studies, and from natural humic material as well in the latter study) enhanced transport of viruses, and they concluded that the effect was due to competition between the viruses and organic matter for sorption sites.

The results summarized here would point to an effect of organic matter on bacterial sorption (and therefore transport), by organic matter sorbed to a substratum and possibly by organic matter in suspension. However, the study remained to be performed under conditions which are controlled, yet somewhat representative of the components found in a natural aquifer. Further, no attempt has been made to determine what effect bacterial surface properties might have on the interaction between cell, organic matter, and substratum. In the current study, a beginning was made toward this goal by using strains of bacteria with different surface characteristics, varying concentrations of dissolved sodium humate in conjunction with clean (and to a lesser extent, iron-coated) sand, and varying concentrations of sodium humate sorbed to iron-coated sand. Work was performed in batch experiments in the laboratory and efforts were made to keep consistent variables which have been seen to have an effect but were not currently of interest, such as growth stage of the cells, ionic strength of the suspension, size of the porous media grains, etc.

In the work described in this paper, the presence or concentration of dissolved sodium humate did not affect bacterial sorption or aggregation. Strain was, however, an important factor in the dissolved humate experiments, with S138 (the more hydrophilic and more negatively charged strain) sorbing to clean quartz sand, and S139 not sorbing to the sand. Both strains were strongly retained by iron-coated sand, but retention on the iron-coated sand decreased with increased sorbed humate concentration. Bacterial sorption to iron-coated sand (with or without sorbed humate) was not affected by strain.

MATERIALS AND METHODS

Bacterial strains. The strains used (S138 and S139) were provided by Dr. David Balkwill of Florida State University from the Dept. of Energy's Subsurface Microbial Culture Collection. Hydrophobicity was determined as the water contact angle (CA) (Bar-Ness and Rosenberg 1989) by placing a drop of distilled water on a thick smear of freshly dried bacteria and measuring the contact angle after the drop had stabilized (had not spread for several seconds). Size of the cells was determined by projecting photomicrographs of pure cultures stained with acridine orange (Hobbie, et al. 1977) and measureing at least 50 cells of each strain with a ruler. Calibration was performed with a projected slide photograph of a stage micrometer. S138 had a CA of 17 ± 3°, a length of 1.0 ± 0.2 μm, and a width of 0.73 ± 0.10 μm. S139 had a CA of 70 ± 0°, a length of 0.92 ± 0.23 μm, and a width of 0.63 ± 0.12 μm. Zeta potentials for the two strains were calculated from electrophoretic mobility measurements, and were -56.3 mV and -15.15 mV for S139 and S138, respectively.

Media and growth conditions. The bacteria were maintained at room temperature on half-strength peptone-yeast extract agar (125 mg peptone, 125 mg yeast extract, 15 mg $MgSO_2 \cdot 2H_2O$, 1.75 mg $CaCl_2 \cdot 2H_2O$, and 15 g agar $liter^{-1}$

of distilled water). For experimental use, strains were grown in half-strength peptone-yeast extract broth for 48 hours, centrifuged at 38,000 $\times g$ for 10 minutes, suspended in artificial ground water (AGW; 1.5×10^{-5} M KNO_3,1.4 $\times$ 10^{-4} M $MgSO_4\cdot2H_2O$, 7.0×10^{-5} M $CaSO_4\cdot2H_2O$, 8.0×10^{-5} M NaCl, and 1.4×10^{-4} M $NaHCO_3$), centrifuged again and resuspended in AGW. The cells were left suspended in AGW for 24 to 48 hours to ensure that they had entered a resting state (Fontes, et al. 1991).

Experimental solutions. In all batch experiments, AGW (pH 6.3-6.6) was used as the suspending solution. The pH did not change upon contact of the AGW with clean or coated sand. For batch experiments using clean quartz sand as the solid phase, a commercial sodium humate salt (Lot #01816HH, Aldrich Chemical Co., Milwaukee, WI) was dissolved in the AGW at concentrations of 0.1 mg liter^{-1} and 1.0 mg liter^{-1}. A control with no humate was also run. The manufacturer's analysis of this lot of sodium humate was 42.18% C, 3.31% H, 0.37% N, 2.02% Na, 0.678% Fe, 0.216% Al, 0.101% Si, 0.07% Ca, 0.0365% Mg, 0.012% Ba, 0.04% K, and 0.0025% or less Sr, Mn, Zn, and B. The mineral cation content of the sodium humate salt should not have had a significant effect on the ionic strength of the AGW. At 0.1 mg liter^{-1}, the ionic strength was calculated to be about 1.7×10^{-6} M, or 0.16% of that of the AGW ($I = 1.079 \times 10^{-3}$ M). The pH of AGW containing dissolved sodium humate also averaged 6.5.

Sand. The sand used for all experiments was Granusil Silica #4095 obtained from Unimin Corp. (Portage, WI). This sand was sieved to obtain a size fraction between 0.60 and 0.71 mm. The sand was then cleaned by stirring it vigorously with 10% nitric acid for two hours, rinsing it 6-8 times (until the water appeared to be clear) with distilled water, stirring it with 0.5 M NaOH for one hour, and again rinsing it with distilled water until the water ran clear. The sand was then dried in a drying oven at 95ºC at least overnight (10-12 h).

Iron-coated sand was produced in batches of 200 to 250 g by adding that amount of clean sand to 400 ml distilled water in which 20 g of $FeCl_3\cdot H_2O$ had been dissolved. The mixture was shaken vigorously, and titrated slowly with 0.5 N NaOH from pH 1.9 to 4.5-4.8. The total volume of NaOH required was about 420 ml. The titrated mixture was allowed to shake vigorously for 24 h. The sand was then rinsed thoroughly with distilled water, dried at 95ºC, rinsed and dried again. Six batches were produced and mixed together. The pH at the zero point of charge (pH_{ZPC}) of sand coated by this process was 6.8 (potentiometric titration, Scott Brooks, personal communication).

To coat the iron-coated sand with humate, a method similar to that used by Parfitt et al. (1977) was used. Iron-coated sand was incubated in a 1:1 solution-to-solid (v/w) ratio with a 0.1 N NaCl solution containing either 24 mg liter^{-1} or 12 mg liter^{-1} Na-humate. The higher concentration was selected because it was the approximate maximum amount that could be sorbed under such conditions (Richardson 1994). The mixture was incubated for 24 h on a rotary shaker (~125 rpm), and A_{300} was measured before and after incubation. The solution was then poured off, and the sand dried overnight at 95°C. After the sand cooled, AGW was added to yield a 2:1 solution-to-solid (v/w) ratio, the mixture was placed on the rotary shaker for 15 min, and the absorbance was measured. This process was repeated four more times to ensure removal of unsorbed humate and NaCl. An average of 82% of the humate for the 12-mg liter^{-1} and 88% for the 24-mg liter^{-1} treatments was retained. Coatings of 9.4×10^{-3} mg humate g^{-1} sand (SD 4.6×10^{-4} mg g^{-1}) and 2.1×10^{-2} mg humate g^{-1} sand (SD 1.2×10^{-3} mg g^{-1}), respectively, were obtained. The humate coating was performed in small batches, which were mixed together to minimize variation between experiments.

Experimental procedures. Prior to the running of each sorption experiment, 125-mL Erlenmeyer flasks were washed in a 10% nitric acid bath for at least 24 h, rinsed with filtered distilled water (FDW), and dried. 51 ml of AGW or AGW + humate were measured into each flask. The flasks and solutions were then autoclaved.

To begin each experiment, an initial concentration of S138 or S139 cells in AGW was determined using the acridine orange direct count (AODC) method (Hobbie, et al. 1977). Each solution was inoculated with approximately 2×10^6 cells and mixed thoroughly. A 1-mL sample was removed to obtain an initial count; afterward, an additional volume equivalent to the volume of the inoculant was pipetted out. This was to bring the volume to 50 ml; however, it was later determined that about 5 ml of solution had been lost during the autoclaving process, so that the final volume was only about 45 ml. After the sample was taken, 25.0 g of sand was added (intended to result in a 2:1 solution:solid mass ratio; in reality, it may have been closer to 1.8:2, but was at least consistent), and the flask was placed on a rotary shaker for three hours. Samples were taken for enumeration by AODC at the end of the three-hour period. Results were expressed as the percentage of cells retained calculated as:

100 $\times$ (final cell concentration in suspension / initial cell concentration in suspension).

RESULTS

Dissolved humate experiment. The more hydrophobic strain (S139) did not sorb appreciably to clean sand, whereas about 50% of S138 cells sorbed across the treatments (Table 1). There was no obvious difference between the humate treatments, with the possible exception of the 0.1 mg liter^{-1} humate treatment for S138, which had greater sorption than any of the others. However, the overall lack of effect of humate concentration was confirmed by the results of an ANOVA which demonstrated the effect of strain to be highly significant ($p < 0.0005$), whereas the effect of humate was not significant at the $\alpha = 0.05$ level ($p = 0.146$).

Additionally, no difference in sorption of the cells to the grains was seen when S138 was combined with iron-coated sand and 1 mg liter^{-1} humate in AGW and compared to a similar treatment without the humate. The retention in the presence of dissolved humate was

98% (SE = 1), while that in the absence of humate was 97% (SE = 1). Although the difference between the clean sand and iron-coated sand is obvious, it is also clear that dissolved humate in concentrations of 1 mg liter^{-1} or less did not affect bacterial sorption.

TABLE 1. Mean percentage retention for cells sorbed to clean quartz sand

	Dissolved Na-humate (mg liter^{-1})[a]		
STRAIN	0	0.1	1
S138	53 ± 2	61 ± 2	37 ± 8
S139	2.0 ± 5.9	-2.0 ±2.8	6.8 ± 3.9

[a]Mean ± SE

Sorbed humate. As seen in Table 2, sorbed humate had a very different effect. Fewer cells sorbed to the iron-coated sand when humate was already sorbed to it, and increasing the humate concentration on the sand surface decreased the sorption even further. The effect of sorbed humate on cell attachment was highly significant ($p < 0.0005$), but the effect of strain was not significant ($p = 0.527$).

TABLE 2. Mean percentage retention for cells sorbed to iron-coated sand with and without humate coating

	Concentration of Na-humate (mg g^{-1} sand)[a]		
STRAIN	0	9.4×10^{-3}	2.1×10^{-2}
S138	94 ± 0	80 ± 3	64 ± 4
S139	98 ± 0	87 ± 1	60 ± 8

[a]Mean ± SE

DISCUSSION

Sorption of bacteria to clean sand. That S139, the more hydrophobic strain, should not sorb to clean sand while S138 did was an unexpected result in light of much of the work done by others. For example, both Stenström (1989) and van Loosdrecht et al. (1987a) found that increased hydrophobicity resulted in increased sorption and that hydrophobicity was more important than surface charge in determining the extent of sorption. Van Loosdrecht et al. (1987a, 1987b) used negatively charged polystyrene with a contact-angle of 70°, making it relatively hydrophobic. Stenström (1989), however, found that sorption increased with cell hydrophobicity even with a hydrophilic substratum such as quartz. On the other hand, Fletcher and Loeb (1979) found sorption of a *Pseudomonas sp*. was lessened considerably on hydrophilic surfaces relative to hydrophobic surfaces. That sorption of a hydrophobic cell would be less with a hydrophilic surface would seem to be borne out by the findings of Absolom et al. (1983).

If the interpretation of the relative hydrophobicity for S138 and S139 is accurate, then one of two conclusions must be reached for the conditions imposed in the experiment: either increased hydrophobicity had an adverse effect on sorption (because of the hydrophilic nature of the substratum) or it was relatively unimportant compared to another factor, such as electrostatic charge.

The relative retentions of S138 and S139 were consistent with expectations based on charge alone. The pH_{zpc} for quartz is 2.0 (Stumm and Morgan 1996). For pH levels above that, quartz should have a negative charge; it would certainly be negatively charged at the pH range involved in these experiments. Since the Zeta potential of S139 was much more negative than that of S138 (-56.3 mV vs. -15.15 mV respectively), DLVO theory would predict that S138 would sorb to a greater extent than S139. That more negatively charged bacteria were retained to a greater extent was also found by Sharma et al. (1985).

One notable difference between studies which have found an electrostatic effect and those which have found a hydrophobic effect is the ionic strength at which they have been run. Studies cited here which have found an electrostatic effect, whether it be due to ionic strength of the suspending liquid (Fontes, et al. 1991, Marshall, et al. 1971, Scholl, et al. 1990) or the surface charge of the bacteria (Sharma, et al. 1985), have used ionic strengths below 0.1 M. Studies which have observed a hydrophobic effect (Absolom, et al. 1983, Fletcher and Loeb 1979, Stenström 1989, Van Loosdrecht, et al. 1987a, Van Loosdrecht, et al. 1987b) have been run at ionic strengths greater than 0.1 M, except for that performed by Mozes et al. (1987), who used distilled water. Gordon and Millero (1984) found that the nature of electrostatic interactions changed at 0.1 M. Below this level, bacterial sorption increased with ionic strength, as predicted by DLVO theory. Above 0.1 M, DLVO theory no longer applied; bacterial sorption was unaffected by ionic strength, but decreased with increasing cation concentration. This may help to explain why electrostatic interactions have been found to be, at best, of secondary importance at higher ionic strengths. It may also serve to explain why Scholl et al. (1990) and Mozes et al. (1987) observed changes in sorption with pH, while Stenström (1989) did not. The ionic strength used in the current study (1.079×10^{-3} M) falls well within the range in which DLVO theory

would be applicable. Since ground water normally exhibits a low ionic strength, it would seem to be more appropriate to use a low ionic strength to simulate sorption in it.

The fact that no effect of dissolved humate was seen on sorption of bacteria to clean sand does not seem surprising in light of the fact that the humate did not sorb to clean sand. However, there was an interaction effect of humate with bacterial strain, and there is also the possibility that sorption of S138 may have actually been enhanced by the presence of 0.1 mg liter^{-1} of dissolved sodium humate (retention in 0.1 mg liter^{-1} humate was 61% ± 2%; the next highest retention was 53% ± 2%, for the treatment without humate). If the humate could not have interacted with the bacteria at the sand surface, any interaction must have occurred in solution. It is difficult to imagine, however, how an interaction with something that has a lesser affinity for sand than the bacterium does would enhance sorption of the bacterium; it would be exactly the opposite result of that found by Rav-Acha and Rebhun (1992) for interaction of humate with hydrophobic organic compounds, and counter to intuition. Further, it seems unlikely that a chemical type known for its hydrophobic interactions would interact directly with a hydrophilic bacterium, but not a hydrophobic bacterium. Given that humate is capable of non-hydrophobic interactions such as hydrogen and van der Waals bonding, though, it may be possible. It must also be acknowledged that commercial humic acid has been found to be a less-than-perfect analogue of naturally-occurring humic acid (Chiou, et al., 1987, Malcom and MacCarthy, 1986); however, commercial humic acids have been found to be even less hydrophilic than average (Chiou, et al. 1987). It may also be that the interaction is indirect, such as a lowering of the surface tension of the water which occurs with nearly all organic substances that occur in natural waters (Powelson, et al. 1990). However, this argument does not explain why the effect would only be seen at a very low concentration.

Sorption of bacteria to iron-coated sand. As in our previous studies (Mills, et al. 1994, Scholl, et al. 1990), coating the quartz sand with iron oxyhydroxide strongly increased bacterial retention. The most obvious explanation for this effect is the alteration of the surface charge of the sand by the iron coating, since coating the sand raises the pH_{zpc} from approximately 2 to about 6.8. This would mean that at the experimental pH range of 6.3 to 6.6, where the clean sand is definitely negatively charged, the iron-coated sand would be neutral or have a slight positive charge. Bacterial strain was not found to be significant with iron-coated sand, which implies that a positive (or circumneutral) charge on the substratum is more important than either the degree of negative charge or the hydrophobicity of the bacterial surface. This might indicate that the sand is actually weakly positively charged, since if it were neutral, hydrophobic interactions might become important, and if it were weakly negatively charged, the degree of negative charge on the bacteria should be important. The lack of strain effect in sorption to iron-coated sand (with two strains that were known to differ in hydrophobicity) was also reported by Mills et al.(1994).

Sorption of bacteria to humate-overcoated sand. Sorbed humate had a definite effect on the sorption of bacteria to iron-coated sand, and that effect was negative. Further, the inhibitory effect was increased with an increase in sorbed humate. This would seem to point to a competition of the negatively charged humate with the negatively charged bacteria for sorption sites on the iron-coated sand (again, making it seem likely that there was a slight positive charge on the sand). Competition for the interaction of organic matter and viruses was hypothesized by both Lance and Gerba (1984) and Powelson et al. (1990). Scholl and Harvey (1992) also considered the possibility of a competitive interaction to explain why sand that was coated both with iron oxyhydroxide and organic matter sorbed fewer bacteria when organic matter was present in the suspension. They did not see the same result with iron-coated sand with the organic matter removed, but suggested that further study with better-defined materials might shed some light on the matter.

If competition is the reason for the decreased sorption of the bacteria, it seems curious that no lowering of sorption was seen in the experiment with S138 and iron-coated sand that included 1 mg liter^{-1} humate in the suspension. This may be due to the small amount of humate in solution in this experiment relative to the amount of humate that was sorbed to the sand in the other experiment. If the relationship of sorbed humate to bacterial sorption could be assumed to be linear, an extrapolation from the sorbed-humate study demonstrates that the bacterial sorption if 1 mg liter^{-1} humate sorbed to the sand is only about 2% less than the sorption seen with no humate present. This would fall within the experimental error.

It is somewhat surprising that a bacterial strain effect was not seen once humate was sorbed to the sand. Presumably, the areas where the humate was sorbed would have become more negatively charged and more hydrophobic. If the assumption is made that, as in the clean sand sorption experiments, charge is more important than hydrophobicity, then it might be expected that S139 would be repelled more than S138. That expectation was not realized, however, possibly

because the effect of the humate may be extremely localized.

CONCLUSIONS

Although organic matter is ubiquitous in natural waters, its effects on transport of bacteria have been little-studied. The results seen in this work indicate that, although organic matter may not be important in transport through relatively clean sand, in iron-coated sand the effect of organic matter cannot be ignored. The high sorption rates seen for bacteria on sand coated with iron hydroxide alone will drop significantly if organic matter is sorbed to the sand, and sorption will continue to decrease with increasing organic matter. The mechanism observed in this study would appear to be a competitive interaction, but future experiments should concentrate on elucidating this more thoroughly, by using differing amounts of dissolved organic matter in addition to, and instead of, sorbed organic matter.

The effect of bacterial surface characteristics was also clearly seen. This effect may be different in the field than that seen in many laboratory experiments, because of the difference in ionic strengths used. In this study, which used an ionic strength more closely approximating that found in the field, it appeared that surface charge was more important than hydrophobicity in determining bacterial sorption to clean quartz sand. However, neither was a factor in determining bacterial sorption to either iron-coated sand or iron-coated sand with a humate overcoat. The relative importance of hydrophobicity and surface charge over a range of ionic strengths would be another fruitful topic for further exploration.

ACKNOWLEDGMENT

This research was funded by the Subsurface Science Program, Office of Health and Environmental Research, U. S. Department of Energy award number DE-FG05-89ER60842. Measures of electrophoretic mobility were provided by Jiamin Wan at the New Mexico Institute of Mining Technology and John McCarthy at Oak Ridge National Laboratory.

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