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Microbial Effects on Colloidal Agglomeration

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DISCLAIMER

MICROBIAL EFFECTS ON COLLOIDAL AGGLOMERATION

by Larry E. Hersman

Abstract

Colloidal particles are known to enhance the transport of radioactive metals through soil and rock systems. This study was performed to determine if a soil microorganism, isolated from the surface samples collected at Yucca Mountain, NV, could affect the colloidal properties of clay particles. The agglomeration of a Wyoming bentonite clay in a sterile uninoculated microbial growth medium was compared to the agglomeration in the medium inoculated with a Pseudomonas sp. In a second experiment, microorganisms were cultured in the succinate medium for 50 h and removed by centrifugation. The agglomeration of the clay in this spent was compared to sterile uninoculated medium. In both experiments, the agglomeration of the clay was greater than that of the sterile, uninoculated control. Based on these results, which indicate that this microorganism enhanced the agglomeration of the bentonite clay, it is possible to say that in the presence of microorganisms colloidal movement through a rock matrix could be reduced because of an overall increase in the size of colloidal particle agglomerates.

I. Introduction and Literature Review

Colloidal dispersion has been implicated as a means of transporting toxic wastes, heavy metals, and radioactive wastes through soil and rock systems (Buddemeir and Hunt 1988; J. McCarthy, Oak Ridge National Laboratory, personal communication). When irreversibly attached to a colloid, these substances are unable to participate in adsorption/desorption reactions with the soil or rock matrix and can potentially move at an accelerated rate with the colloids through the soil or rock matrix. If, however, colloids become attached to one another to form agglomerates, then the colloids would no longer be available to participate in colloidal dispersion processes because their increased size would exclude them from movement through the small pores. Obviously, the net result of this colloidal agglomeration would be an overall decrease in the transport of metals and wastes. With that supposition, we investigated the influence of bacteria upon the agglomeration of clay colloids.

Within the literature there is a substantial body of information regarding the interactions between microorganisms and colloidal particles. Those interactions include the attraction processes, adhesion, adsorption, and flocculation.

A. *Attraction of Bacteria to a Solid Surface*

Many natural habitats have a low nutrient status; therefore, solid surfaces are potential sites for concentrating nutrients (as ions and macromolecules) and, consequently, for promoting intensified microbial activity. The movement of water across a surface provides increased opportunities for microorganisms to approach solid-liquid interfaces. In addition, there are several physico-chemical and biological attraction mechanisms operative in the immediate vicinity of interfaces:

1) *Chemotaxis.* Mobile bacteria are capable of a chemotactic response to low concentrations of nutrients introduced into a normally nutrient-deficient system. Chemotaxis, of course, cannot account for the attraction of nonmotile bacteria to solid-liquid or other interfaces.

2) *Brownian motion.* Although more of a collision process, Brownian motion (colloidal particles in a state of continual random motion caused by the chaotic thermal motion of molecules) can cause molecules to collide with each other and with particles suspended in the liquid.

3) *Electrostatic attraction.* The interaction of negatively charged surfaces of bacteria with solid surfaces probably depends on the properties of the surface in question. Because most surfaces in nature are negatively charged, it is unlikely that electrostatic phenomena are involved directly in the attraction of bacteria to such surfaces. It should be emphasized, however, that solids in natural environments often acquire different surface properties through sorption of macromolecule compounds to the surface. Therefore, the surface, as "seen" by the bacteria, may be very different from the original surface.

4) *Van der Waals interaction.* "This interaction involves weak bonding between polar units, either permanent (like OH and C=O) or induced by the presence of neighboring molecules. The induced Van der Waals interaction is the result of correlations between fluctuating polarizations created in the electron configurations of two nearby nonpolar molecules. Although the time-averaged polarization induced in each molecule is zero (otherwise it would not be a nonpolar molecule), the correlations between the two induced polarizations do not average to zero. These correlations produce a net attractive interaction between the two molecules" (Sposito 1989). Although the Van der Waals interaction between just two molecules is very weak, the Van der Waals component is additive and strong when many molecules or larger surface areas interact simultaneously .

5) *Electrical double-layer effects.* When two negatively charged bodies are in close association, they may be held close to each other by a connecting bridge of cations in solution, called the double layer. The strength of the bonding between the two particles, through the double layer, depends on the thickness of the interacting aqueous double layers, which in turn, is dependent on the concentration and the valency of the electrolyte (cations).

6) *Cell-surface hydrophobicity.* It is quite reasonable to assume that part, or all, of the outer surface of some bacteria is hydrophobic. It is, therefore, reasonable to consider that such bacteria are rejected from the aqueous phase and attracted towards any nonaqueous phase, including solid surfaces.

As stated earlier, surfaces are the potential sites of nutrient concentration, and consequently of increased microbial activity. Adsorption of organic substrates on soils depends on the nature of the particulate matter, the organization of the fabric, the clay types, the catioic status of the soils, and the concentration and molecular structure of the substrate. Hence, the availability of substrates to soil microorganisms may be enhanced or reduced by the presence of particulates. If substrates are concentrated at the surface of clay minerals, then these minerals would become populated with microorganisms using those substrates, thereby increasing the potential for interactions between microorganisms and clay particles. It is therefore important to discuss substrate sorption by these particles.

Sugars sorb poorly to clays (Greenland 1956). Metabolism of glucose in soil is inversely related to the degree of bacterial sorption, which is related to the participating bacterial species (Dianowa and Weroschilowa 1925) and the soil cation status (Peele 1936). Stotzky (1966a,b) and Stotzky and Rem (1960,1966) determined that kaolinite had little effect on bacterial respiration with glucose as a substrate, whereas montmorillonite significantly stimulated respiration. Novakova (1972a,b) showed that the sodium and calcium forms of a montmorillonitic clay stimulated glucose decomposition, but that these forms of kaolinite were inhibitory. Stotzky (1966a) also reported that when samples of montmorillonite were made homoionic to a range of cations, there was increased stimulation of bacterial respiration with saturating cation in the order Na >Ca >Mg >K >H. In a separate study, Stotzky (1966b) reported that bacterial respiratory activity was also related to the cation-exchange capacity and surface area of clays, but not to their particle size.

Many other excellent studies have been performed to determine the effects of clays on the decomposition of starch (Filip 1973; Olness and Clapp 1972), aldehydes (Kunc and Stotzky 1970), pesticides (Hance 1974; Weber and Coble 1968), and protein (Filip 1973; Harter and Stotzky 1973; McLaren 1957). In most of these studies, the effect of the clay depended upon the clay type and the ionic nature of the solvent. In fact, these parameters appear to dominate the effects of clays on microbial activity.

B. ***Adhesion***

The adhesion of bacteria to inanimate surfaces is widely recognized as having enormous ecological significance. Adhesion of microorganisms is involved in certain diseases of humans and animals, in dental plaque formation, in industrial processes, in fouling of

man-made surfaces, in microbial-influenced corrosion, and in syntrophic and other community interactions between microorganisms in natural habitats. Most aquatic bacteria appear to adhere to surfaces by means of surface polymers, including lipopolysaccharides, extracellular polymers and capsules, pili, fimbriae, flagella, and more specialized structures such as appendages and prosthecae. Even though these surface components play a role in the initial, reversible adhesion, they often serve to anchor the bacterium at an interface by polymeric bridging.

The composition and quantity of bacterial cell surface polymers vary considerably and are strongly influenced by growth and environmental conditions. Although extracellular polysaccharides have been reported as being responsible for irreversible adhesion, this is not always true. For example, Brown et al. (1977) demonstrated adhesion in mixed, carbon-limited populations despite no evidence of extracellular polymer production. Also, a nitrogen-limited culture resulted in poor adhesion, although large extracellular polymer production was observed. It should always be kept in mind that polymers present between the cell and the substratum but not observed in light or scanning electron microscopy could be responsible for the adhesion. It appears that, with respect to inanimate surfaces, there is a subtle balance between cell surface components [extracellular polysaccharides or lipopolysaccharides (LPS)] that may reduce or promote (fibriae) adhesion to inanimate surfaces. Jonsson and Wadstrom (1983) demonstrated that an encapsulated Staphylococcus aureus strain did not bind to hydrophobic Octyl-Sepharose gel, whereas a noncapsulated variant showed binding capabilities. In fact, polyanionic extracellular carbohydrate material may not be of primary concern with the initial adhesion processes, but rather with development of subsequent bacterial film (Pringle et al. 1983). The presence of LPS reduces cell surface hydrophobicity and decreases adhesion to the air-water interface of Salmonella typhimurium (Hermansson et al. 1983).

At short distances, ionic and dipolar, H-bonds, and hydrophobic interactions are important. Therefore, it is possible that the variabilities in bacterial surfaces result in various types of interactions that occur simultaneously with a single bacterial type. For example, hydrophobic interactions adjacent to ionic or hydrogen bonds can stabilize an otherwise energetically weak binding complex (Hermansson et al. 1983).
Doyle et al. (1982) propose that adhesion can be described in terms of positive cooperation; i.e., once the initial bacterium is bound, compounds on the substratum may change conformation, creating new receptor sites for other cells. The formation of new

sites could be the result of the influence of hydrophobic interactions, but enzymatic action exposing saccharide receptors or cell-cell binding at the high bacterial concentrations used should also be considered. Unfortunately, theories on specificity in adhesion at inanimate surfaces and the ecological significance thereof must be discussed in view of behavior that is often very system-specific.

C. *Adsorption of Colloidal Clay*

The adsorption of colloidal clays to bacterial surfaces has been studied by Lahav (1962) and Marshall (1968, 1969a,b). Lahav (1962) suggested that clay platelets may be oriented in a number of ways at the bacterial surface, as a consequence of the net negative charge on clay platelets and the existence of some positive charges on broken edges of platelets. Marshall (1968,1969b) reported that a species of Rhizobium with a carboxyl type ionogenic surface sorb more Na+- illite per cell than do species with a carboxyl-amino ionogenic surface. Using sodium hexametaphosphate (HMP) to suppress positive charges on platelet edges, Marshall (1969a) found that the HMP-clay did not sorb to a carboxyl type bacteria, whereas a limited amount of this clay sorbed to carboxyl-amino type bacteria. He interpreted these results in terms of the electrostatic attraction between the platelets and the bacterial cell surfaces. Normal sodium montmorillonite particles sorb in an edge-to-face manner to carboxyl type bacterial surfaces, with positively charged edges of the clay attracted to the negatively charged bacterial surface. Sorption in this manner is prevented by neutralization of positive edge charges of the clay by HMP.

D. *Microbial Flocculation of Clays*

In recent years, several processes have been patented for the flocculation of clays, particularly clays derived from phosphate beneficiation. Microbial polysaccharides from such organisms as Pullularia, Xanthomonas, Arthrobacter, Cryptococcus, Hansenula, and Plectania were found to flocculate finely divided inorganic solids in an aqueous medium (Goren 1968). In another application (Bomstein 1972), the use of alkaline-treated microbial nucleoprotein is described for flocculating organic and inorganic wastes. Used in this process were nucleoproteins from the microbes Polangium, Myxococcus, Sorangium, Flavobacterium, Leuconostoc, Micrococcus, and Alcaligenes. Nucleoprotein derived from these organisms was treated with any one of a variety of alkaline compounds, including $Ca(OH)_2$, KOH, NaOH, NH_3, Na_3PO_4, and quaternary ammonium compounds, which would raise the pH to the point where the microbial material would lyse and form a Sol. Flocculation of suspended waste resulted when the

concentration of alkaline-treated microbial material was present in concentrations of 1 to 500 ppm (Bomstein 1972). Floc deterioration can result from biological factors as well as physical factors. Synthetic and natural polymers used for flocculation of colloids may be subject to degradation by microorganisms, which could result in floc destabilization (Brown and Lester 1979; Obayashi and Gaudy 1973).

As can be seen from the above discussions, there are several ways in which bacteria and clay particles may interact, including attraction processes, adhesion, adsorption, and flocculation. It is entirely possible that such interactions could affect the distribution of individual particles in solution. We therefore performed laboratory experiments to investigate the interaction of bacterial and clay particles and its effect on clay particle distribution. The primary focus of these experiments was to determine if bacteria are able to influence significantly the agglomeration rate of colloidal particles. Two separate experiments were performed; the first determined if the presence of bacterial cells affected colloidal agglomeration, while the second examined the effects of extracellular bacterial metabolic products, in the absence of bacterial cells.

II. Materials and Methods

A. *With Cells*

Sterile Wyoming bentonite clay (approximately 0.2 µm diameter, 0.05 g) was added to 100 ml of sterile water. The suspension was mixed, and 1.0 ml of the suspension was added to 20 ml of nutrient broth. Three flasks containing the 20 ml nutrient broth/colloidal suspension were inoculated with a Pseudomonas sp., which was isolated from surface samples collected from Yucca Mountain, NV. Three of the same flasks remained uninoculated, and three flasks void of colloids were inoculated with the same bacteria. One flask void of colloids remained uninoculated, serving as a sterile control.

With an Olympus Vanox microscope, these suspensions were monitored for over 200 hours. Using phase contrast microscopy, it was very easy to differentiate the bacteria from the clay particles. Individual colloids and agglomerates (clusters) containing 2 to 5, 6 to 1O, 10 to 25, and >25 particles, were counted. Counts were made with a Petroff-Hausser bacteria counting chamber having a volume of 1/400 mm^2 by 1/50 mm deep, or 5 x 10-5 mm^3, or 5 x 10^{-8} ml. This experiment was repeated three times, and the results are presented in **Figures 1-8** as counts per 5 x 10^{-8} ml. At the end of the experiment, the

final pH of the inoculated flasks was approximately 8.6, as compared to a initial pH of 7.0.

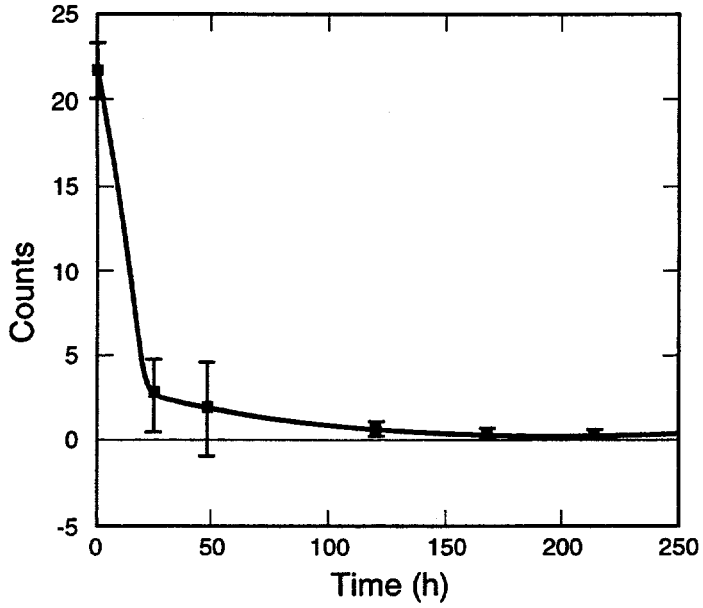

Fig. 1. Number of individual clay particles per 5.0×10^{-8} ml in the presence of a *Pseudomonas* sp.

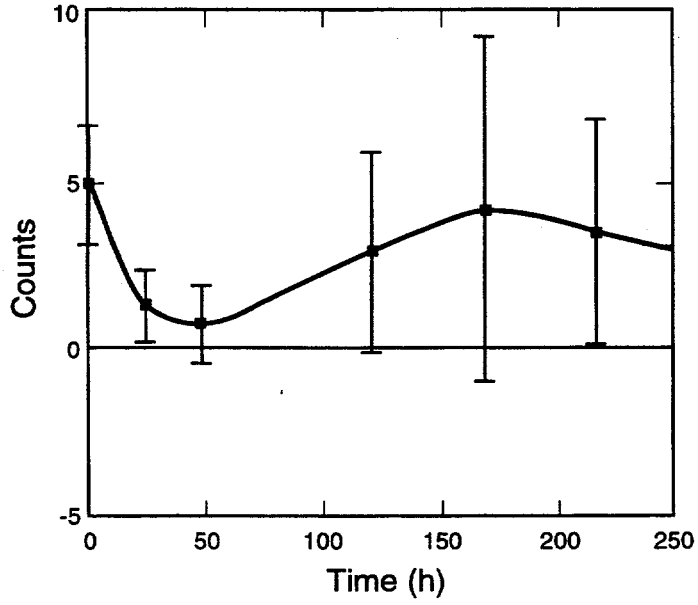

Fig. 2. Number of clusters per 5.0×10^{-8} ml, containing 2 to 5 clay particles, in the presence of a *Pseudomonas* sp.

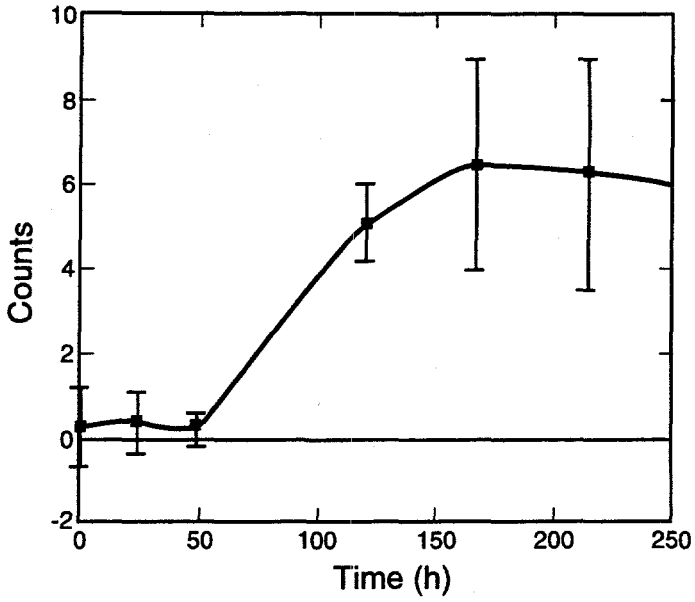

Fig. 3. Number of clusters per 5.0 x 10^{-8} ml, containing 6 to 10 clay particles, in the presence of a *Pseudomonas* sp.

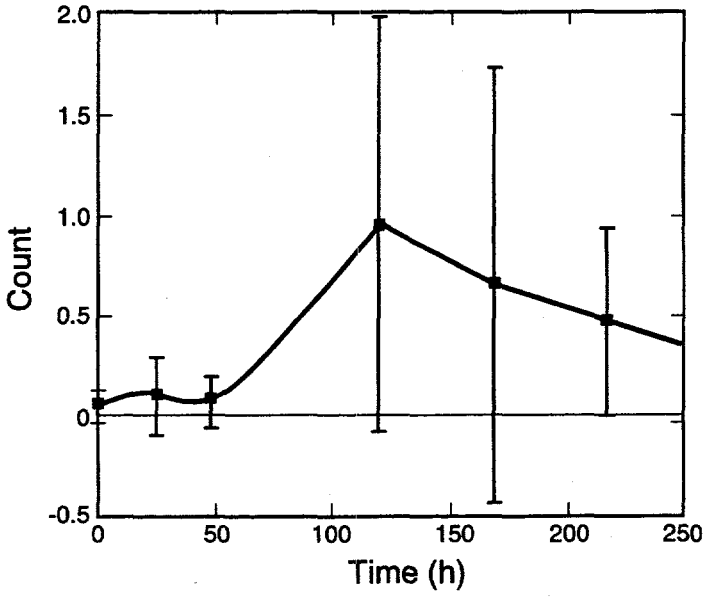

Fig. 4. Number of clusters per 5.0 x 10^{-8} ml ml, containing 10 to 25 clay particles, in the presence of a *Pseudomonas* sp.

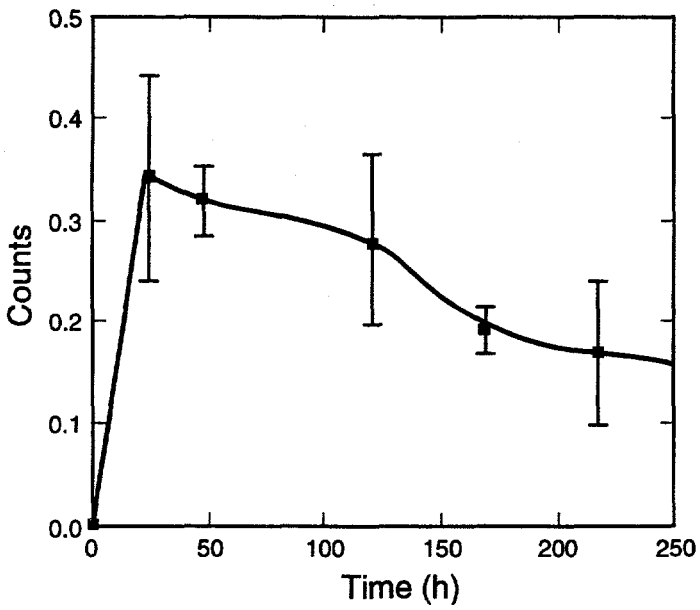

Fig. 5. Number of clusters per 5.0×10^{-8} ml ml, containing greater than 25 particles, in the presence of a *Pseudomonas* sp.

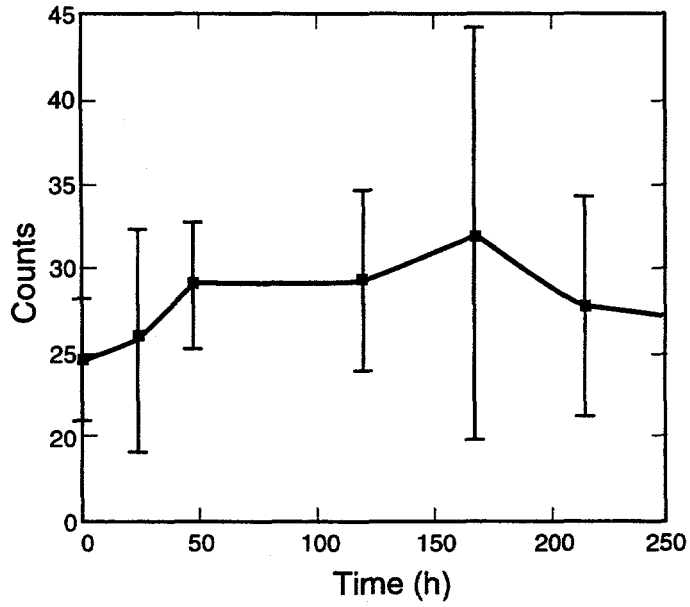

Figure 6. Number of clay particles per 5.0×10^{-8} ml, in sterile nutrient broth.

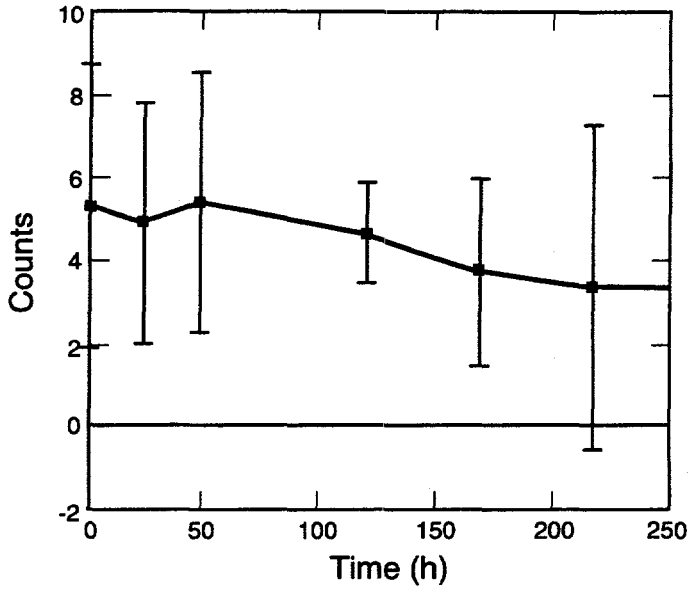

Figure 7. Number of clusters per 5.0 x 10^{-8} ml, containing 2 to 5 clay particles, in sterile nutrient broth.

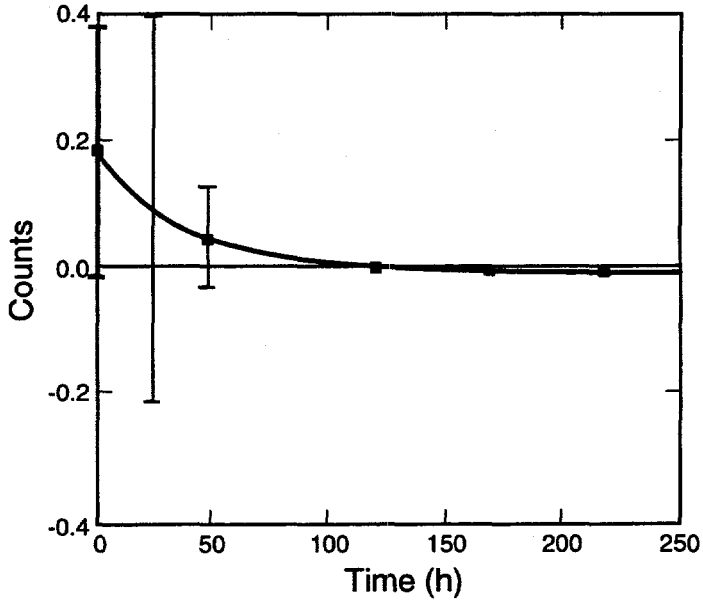

Figure 8. Number of clusters per 5.0 x 10^{-8} ml, contain 6 to 10 clay particles, in sterile nutrient broth.

B. *Without Cells*

One liter of succinate media, composed of the following ingredients, was prepared.

Ingredient	**g/L**
K_2HPO_4	0.091
NH_4Cl	1.00
$MgSO_4.7H_20$	0.0625
$CaCl_2$	0.05
Succinic Acid	1.00

The growth medium included the following ingredients: (g.l-1): K_2HPO_4, 0.091; NH_4Cl, 1.0; $MgSO_4.7H2O$, 0.063; $CaCl_2$, 0.05; $MnSO_4.H_2O$, 6.25 x 10-6; $CuSO_4$, 5.38 x 10^{-6}; $CoSO_4.7H_2O$, 4.01 x 10^{-6}; $ZnSO_4$, 4.20 x 10^{-6}; MoO_4, 3.00 x 10^{-6}; succinate, 1.0; casamino acids, 0.125; H_2O, 1.0 l; pH = 6.85.

The medium was filtered sterilized (0.2 μm) and 250 ml was poured into each of 4 sterile 500-ml flasks. Two of the flasks were inoculated with the Pseudomonas sp., while the other two flasks remained as uninoculated controls. The flasks were incubated at room temperature (22°C) for 50 hours, while shaking at 100 **RPM**. The final pH of the medium was approximately 8.5, as compared to an initial pH of 6.8. The cells were removed by centrifugation (8150 x G for 1 h), and the pellets in the inoculated flasks were discarded. The supernatants of the inoculated media (or spent media) and uninoculated controls were saved for later use. Clay particle-size analysis was performed as described by Jackson (1956). Water was added to 10 g of bentonite clay, bringing the volume to 200 ml. The mixture was shaken vigorously until all the clay was in suspension. Following sterilization by autoclave, the cooled clay suspension was removed by centrifugation (200 x G for 3 min). This centrifugation removed particles having a diameter greater than 2.0 μm. The pellets were discarded, and the supernatant was divided in half and placed in a centrifuge (8155 x G for 30 min). These supernatants, containing particles less than 0.2 μm in diameter, were discarded. The bentonite pellets, containing particles between 0.2 and 2.0 μm in diameter, weighted approximately 4.0 g. One of the bentonite pellets was resuspended with the spent media and the other with sterile media. The mixtures were shaken for 12 h at 100 rpm, then the particles were removed by centrifugation (1550 x G for 30 min). The supernatants were decanted, and their absorbance was measured at 500 nm using a Perkin-Elmer Model Lambda 3B spectrophotometer. The experiment was repeated three times and the results are presented in Table I.

Table I. Absorbance (550 μm) comparisons of sterile succinate media, sterile media with clay, spent media, and spent media with clay. "Reference" is the reference light path, while "sample" is the light path containing the sample.

Reference	Sample	Average	Standard Deviation
1. Sterile	Sterile w/clay	0.225	0.046
2. Spent w/clay	Spent	-0.007	0.028
3. Spent	Spent w/clay	0.007	0.028
4. Sterile	Spent	0.024	0.011
5. Spent w/clay	Sterile w/clay	0.204	0.051
6. Sterile w/clay	Spent	-0.209	0.051
7. Sterile	Spent w/clay	0.061	0.103

III. Results and Discussion

A. *With Cells*

These results clearly demonstrate that under the conditions of this experiment, the presence of bacteria significantly influenced the rate of colloidal agglomeration. As can be seen in Fig. 6, in the presence of bacteria, the number of individual clay particles decreased rapidly with time and in the absence of bacteria (sterile), the number of individual clay particles increased with time, presumably because of the disruption of preformed agglomerates present at time O. Both the 2 to 5 and 6 to 10 particle clusters tended to increase with time in the presence of bacteria, while the 10 to 25 and >25 particle clusters followed a more complicated function, first increasing and then decreasing with time.

Sterile clay suspensions followed a more predictable pattern. Generally, the preformed cluster groups (both 2 to 5 and 6 to 1O) tended to disassociate with time, as indicated by the increase in single particles (Figure 6). The inoculated, noncolloid flasks displayed a fairly typical growth curve (results are not presented). The sterile control remained sterile throughout each of the experiments.

From these results, which are extremely important to the YMP Project, one can clearly see that the presence of bacteria profoundly affected the distribution of particles in

suspension. As previously mentioned, colloidal dispersion has been implicated in the transport of radioactive wastes in soil systems, and microorganisms (both indigenous and exogenous) will exist in the vicinity of the candidate site of the potential high-level nuclear waste repository. The results of this study suggest that an interaction could occur between these bacteria and colloidal particles, and as a consequence, colloidal dispersion of actinide elements could be influenced by the agglomeration of colloidal particles.

B. *Without Cells*

In the spent media with clay, there were fewer particles in suspension following centrifugation than in the sterile media with clay. When examining comparison #5 in table I, we observe that the adsorbance of the spent media with clay was less than the sterile media with clay. This indicates that the spent media, containing extracellular metabolic products, increased the mass of the clay particles, either by promoting agglomeration or by binding to individual particles to the extent that their mass was increased, resulting in increased sedimentation by centrifugation. Regardless of the mechanism (agglomeration or increased mass), the effect was the same, a greater amount of clay sedimentation.

The other comparisons in table I serve to demonstrate that there was little difference between the sterile media and spent media (approximately 0.024 absorbance units). There was also little difference between the spent with clay and spent medium (#2), suggesting that few clay particles remained in suspension.

It is important to note that for both experiments the pH of the media changed with time, going from near neutrality at the beginning of the experiments to greater than a pH 8 at their completion. Although this pH change did not pass through the point of zero charge (PZC) of the clay (which is below pH 5.0) it is entirely possible that the effects observed in these experiments could also be due to changes in the surface properties of the clay brought in water chemistry.

The results of these two experiments demonstrate that microorganisms have the potential to enhance colloidal agglomeration. Perhaps by direct contact (with cells), by polymeric interactions (without cells), or by promoting chemical changes in the medium (e.g. pH) the microorganisms significantly increased the agglomeration of Wyoming bentonite clay. This study is supported by much of the information presented in the

introduction section, particularly in sections 1.3 (Adsorption of Colloidal Clay), and 1.4 (Microbial Flocculation of Clays). The increase in agglomeration is in agreement with the discussions of increased clay flocculation rates and the strong potential for clays to be sorbed by microorganisms.

It was, however, beyond the scope of this study to investigate the specific mechanisms of colloidal agglomeration. Rather, the intent of was to investigate the potential for microorganisms isolated from soil samples collected at Yucca Mountain to participate in clay interactions. Based on these results one can state that in the presence of microorganisms, colloidal movement though a rock matrix could be reduced due to an overall increase in the size of colloidal particle clusters.

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