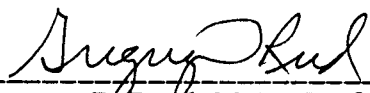


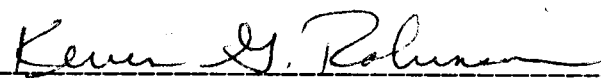
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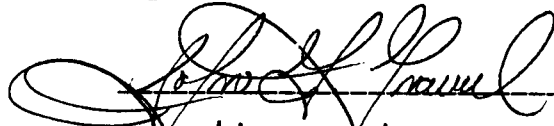
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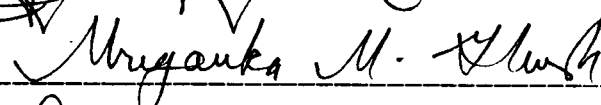


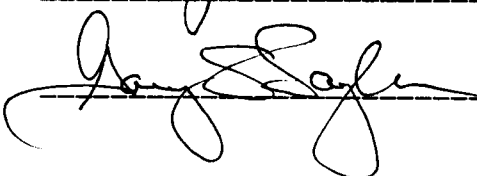
Gregory D. Reed, Major Professor

We have read this dissertation
and recommend its acceptance:









Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

**SOME EFFECTS OF CLAY PARTICLES ON THE MICROBIAL
DEGRADATION OF NAPHTHALENE**

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Rebecca Mirsky Pullen
August 1992

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As I sit down to write this final page, there come to mind so many people that have made both large and small contributions to the successful completion of my doctoral research program. In acknowledging those contributions, I begin with the burden of knowing that, while recognizing those who come to mind immediately, I will be leaving out so many others whose influences have subtly and indelibly made their mark on my life.

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ABSTRACT

Mineralization and bioluminescence assays were used to examine the effects of clay particles on the microbial degradation of naphthalene in aqueous suspensions. An abiotic study of the ability of six different clays to sorb naphthalene led to the selection of a low surface-area kaolinite and a high surface-area montmorillonite to be used in the biological studies. Both of the selected clays demonstrated negligible sorptive capacities for naphthalene. In 12-hour batch mineralization assays using the *Pseudomonas* isolate 5R, the rate and extent of naphthalene mineralization was observed to be retarded in suspensions containing 44% kaolinite when compared with suspensions containing 17% kaolinite or 17% montmorillonite. In a series of bioluminescence assays using the *Pseudomonas* reporter strain HK44, a characteristic light response was identified in which increasing the concentration of clay in the suspension from 0 to 17% had the effect of decreasing the magnitude and the rate of light production. The effect of the clay was determined to be due to factors other than interference with the light detection method. The inhibitory effect of the clay was found to be similar in both mineralization and bioluminescence assays, although after an initial exposure period, light output appeared to be independent of metabolic rate. Also, reproducibility of the results from bioluminescence experiments was highly dependent on the cell growth procedure.

Negative findings were made concerning possible rate-limitations due to adsorption, boundary layer mass transfer, and

intraparticle diffusion. The inhibitory effect was not observed when direct clay-microbe contact was eliminated by using cells immobilized in alginate beads. On the basis of these observations as well as certain assumptions concerning the transport of naphthalene into the cell, a conceptual model has been proposed in which attachment of the cells to clay particles results in blockage of diffusion sites on the cell membrane and a decrease in the rate of naphthalene uptake. The implications of these findings regarding operation of slurry-based bioreactors, and future use of bioluminescent reporter technology in treatability studies are discussed.

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1.0 INTRODUCTION

A unique aspect of research concerned with hazardous waste remediation is the need for the collaboration of many different disciplines. Specifically, the field of environmental biotechnology requires a cooperative interaction among molecular life scientists, ecologists and engineers (Sayler and Fox, 1991). The interdisciplinary approach is absolutely necessary for the successful remediation of contaminated soils. Soil, far from being an inert material like cement or ash, is a heterogeneous multitude of plants and organisms surrounded by an infinitely varying landscape of gaseous, liquid and solid materials. Soils can usually be easily stripped of most contaminants, but the challenge lies in being able to restore a site to its original, productive state. The environmental biotechnology approach to soil remediation, which seeks to achieve the goal of restoration, is constructed on a foundation of molecular biology, genetics, microbial ecology, soil sciences, and chemical and environmental engineering.

One technology that has successfully been applied to treat contaminated soils is slurry-phase bioremediation (Yare, 1991). The slurry-based system has been employed to facilitate contact between contaminated soil particles and microorganisms (Black *et al*, 1991). Knowledge of microbial degradative activity in soil-water systems is also important for understanding the fate of contaminants in subsurface environments (Mihelcic and Luthy, 1991).

Several recent laboratory studies have focused on the biodegradation of organic compounds in aqueous soil slurries (DiGrazia,

1991; Korde, 1991; Mihelcic and Luthy, 1991; Miller and Alexander, 1991). An unexpected observation made by Korde (1991) was that the extent of microbial mineralization was correlated inversely ($r = -0.98$) with the percent clay in four different soils. This finding would certainly have been of interest to Black and coworkers (1991) who, in their slurry-based biotreatment study, found that most of the hydrocarbon contaminants were associated with the fine soil particles, including the clay fraction. Clearly, from the standpoint of the successful operation of slurry-based systems, and for biological treatment of subsurface contaminants, more information is needed regarding the effects of clay particles on biodegradative activity.

Approximately 25 years ago, several studies were made concerning the effects of clays on the metabolism of soil microorganisms (Marshall, 1968 and 1969; Stotzky, 1966a and 1966b; Stotzky and Rem, 1966). In each of these studies, it was recognized that microbial activity is influenced significantly by the presence of clays, but the limitations of the available experimental techniques prohibited the identification of specific mechanisms. Today, the technology is available whereby some of the earlier studies can be repeated and enhanced by using radiolabeled compounds, genetically engineered organisms, and a recently developed bioluminescent reporter assay. These resources and techniques have been employed in this research to increase understanding of microbial degradative activity in soil-water systems by specifically examining the effects of clay particles on the microbial degradation of naphthalene.

1.1 Research Objectives

The overall objective of this research was to examine the effect that clay particles had on the rate and extent of the microbial degradation of naphthalene in an aqueous suspension, and further, to study which mechanisms may be important factors.

More specific objectives included the abiotic characterization of the ability of different clays to sorb naphthalene under various conditions of pretreatment; the performance of bioluminescence assays specifically designed to examine various mechanisms thought to be rate-limiting; and the correlation of bioluminescence and mineralization data.

2.0 BACKGROUND

2.1 Biodegradation as a Remediation Option

Of the various technologies available today for remediation of contaminated soils, one that is receiving close attention is biological treatment. Both naturally occurring and genetically engineered microorganisms have been shown to successfully reduce concentrations in soil of a wide variety of organic contaminants (Rochkind *et al*, 1986). The potential advantages of the biological approach to hazardous waste treatment include reduced cost, reduced risk of exposure, reduced residual contamination, and minimal environmental impact and liability (Skinner *et al*, 1991). However, because many of the processes involved in *in situ* applications of biodegradation are poorly understood, field scale bioremediation frequently does not take place at the rate or to the extent predicted by lab or pilot scale studies. In their natural habitats, microbial populations are diverse collections of different organisms at various stages of growth. The natural system is extremely dynamic and often appears to be chaotic, with small changes in environmental conditions producing large shifts in the ecological stability of the microbial domain. In contrast, laboratory research on the characteristics of microbial decomposition primarily utilizes pure cultures under strictly controlled environmental conditions. The problems associated with successfully duplicating these highly specific laboratory results in the field are therefore numerous and often overwhelming.

2.1.1 Naphthalene Biodegradation

One class of compounds that has attracted much attention in biological treatment studies is the polynuclear aromatic hydrocarbons, (PAH's). PAH's occur in nature as a result of synthesis by some plants, and from naturally occurring forest or prairie fires. However, by far, the largest quantities of PAH's released into the environment come from anthropogenic sources such as fossil fuel burning, coal gassification, and agricultural fires (Edwards, 1983). PAH compounds contain two or more fused benzene rings, and most are practically insoluble in water (eg. naphthalene, 30 mg L⁻¹; phenanthrene, 1.6 mg L⁻¹; anthracene, 0.075 mg L⁻¹; pyrene, 0.14 mg L⁻¹; USEPA, 1980). Plant leaves may adsorb or assimilate PAH's and provide access to the animal food chain where biological activation by enzymes may form carcinogenic and mutagenic epoxides. The potential to permanently eliminate PAH contaminants through microbial mineralization without requiring extensive human exposure is viewed as a significant advantage.

Naphthalene has been chosen for use in several biodegradation studies as a representative PAH (DiGrazia *et al*, 1990; DiGrazia, 1991, Korde, 1991). Bacterial strains have been isolated that completely mineralize naphthalene to carbon dioxide and water by way of a well characterized degradative pathway (Cerniglia, 1984; Gibson, 1971). The microbial attack on naphthalene requires oxygen - the first step in the pathway is the incorporation of both atoms of molecular oxygen into the ring (Cerniglia, 1984). It is believed that the genes responsible for naphthalene degradation are continuously active at a low (or constitutive) level, while the presence of salicylate, a metabolic

byproduct of naphthalene degradation, fully induces the pathway (Schell, 1985).

Due to its hydrophobicity, and therefore its affinity for the similarly hydrophobic cell membrane, microbial uptake of naphthalene is assumed to occur by passive transport. This mechanism allows diffusion of the substrate into the cell as long as the concentration within the cell is less than that in the surrounding medium. The uptake rate is dependent on the concentration gradient, and will decrease with increasing concentration inside the cell (Schlegel, 1985). Factors that alter the concentration gradient in the vicinity of the cell, or those that have an effect on the membrane's transport ability may limit microbial degradative potential. More specifically, it has been proposed that attachment of microbial cells to surfaces may decrease the cell surface area which takes part in substrate uptake, and thus decrease metabolic activity (Hattori and Hattori, 1987; Jeffrey and Paul, 1986; Kieft and Caldwell, 1984).

2.2 The Soil Environment

Soils are heterogeneous and chemically active. Their separate constituents - sand (diameter $> 50 \mu\text{m}$), silt ($2\text{-}50 \mu\text{m}$), clay ($< 2.0 \mu\text{m}$), and organic matter in various stages of decomposition - have a wide array of chemical and physical properties that are influenced by interactions with surrounding liquid and gaseous phases. Stotzky (1972) describes the soil as a vast collection of microhabitats composed of aggregates of sand- and silt-sized particles coated with clay minerals and organic matter, and surrounded by thin films of water. Each microhabitat is a

dynamic system, in a state of constant change such that an alteration in one environmental factor causes simultaneous and subsequent changes in the surrounding microcosm. The task of studying and analyzing soil processes is therefore monumental, and is usually made more manageable by separating components and examining them individually. One of the most often heard criticisms about this approach to soil microbiology research is that the artificial system under observation has no relation to a true soil environment (Stotzky, 1972). While this is a valid point, the difficulties in isolating, preserving and experimenting with soils without excessively changing the very characteristics being studied appear to be insurmountable at this time. Nevertheless, knowledge about individual components of a system is important and valid when viewed as such, and can promote understanding about the system as a whole. It must be stressed however, that observations made from such a reduced system cannot be extrapolated back to the whole system without further study.

2.2.1 Clay as a Soil Constituent

Their charged nature and large surface areas make clays the most active inorganic soil constituents. There are several important characteristics of clay mineral surfaces which contribute to reactions with organic molecules. The cation exchange capacity affects coulombic interaction with cationic organic compounds, the density of the charge on the mineral surface affects the orientation and amount of organics in the reaction, the valence of the cation saturating the mineral exchange complex affects capacity of the surface to react with

organic molecules as well as the strength of mineral-organic bonds, and the expandibility of the mineral affects the amount of surface area exposed to solution (Harter, 1977). Additionally, clay particles effectively retain water films, rendering them more suitable than larger sized sand and silt particles for maintaining microbial populations (Stotzky, 1972). In general, most microbial activity in soil is believed to be associated with the clay mineral fraction (Stotzky, 1972), although the macrohabitat has some influence over this. In some cases, inhibition of aerobic microbial activity in clay environments can be attributed to the dense nature and low permeability of packed clays, and their subsequent resistance to the transfer of oxygen and nutrients (Stotzky, 1972). In a recent survey of subsurface environments, high clay-containing samples yielded few viable microorganisms in comparison with more sandy aquifer material (Beloin *et al*, 1988; Sinclair and Ghiorse, 1989).

Clay minerals are comprised of layers of silicon and aluminum/magnesium sheets in specific ratios. The physicochemical properties of clays are a direct result of their mineralogical composition. Isomorphous substitution, or substitution of one element for another in the mineral structure, can lead to localized charge differences which affect the attractive forces between clay surfaces and other molecules (Bohn *et al*, 1985). The overall effect of isomorphous substitution is to produce a net negative surface charge. This phenomenon plays an important role in the 2:1 (silica-alumina-silica) clays such as smectites, contributing to their high adsorptive capacity. In addition, smectites have relatively weak interlayer

bonding, allowing water to enter between the lattices and cause swelling, a process by which additional sorptive surfaces are created. In contrast, most of the sorption sites of 1:1 (silica-alumina) clays such as the kaolinites are located at the edges of the mineral layers. Overall sorptive capacity is therefore highly dependent on mineralogy.

The presence of oxides of iron and aluminum on clay surfaces can also have a marked effect on sorptive properties by providing local positively charged sites on an otherwise negatively charged surface (Schnitzer and Khan, 1972). Estes and co-workers (1988) found that sorption of low molecular weight halocarbons to montmorillonite (a smectite) was sensitive to changes in pH and ionic strength. This sensitivity was attributed to involvement of surface mineral oxides in electrolyte shielding of sorptive sites. The shielding effect was noted even with small quantities of oxide coating.

2.3 Rate-limiting Mechanisms for Biodegradation in Soil-Containing Systems

At this point, it is necessary to make the distinction between a substance that is biodegradable, and one that is bioavailable. A compound may be considered bioavailable when it can be freely transported across the cell membrane for intracellular metabolism, either as a complete molecule, or after attack by extracellular enzymes (Verschueren and Visschers, 1988). The soil matrix influences bioavailability by providing alternate fates which compete with biodegradation for substrate molecules. It is generally assumed that in a soil-water matrix, a compound must exist in the solution phase in order

for biodegradation to occur (Mihelcic and Luthy, 1991; Stotzky, 1972). Olness and Clapp (1972) suggested that the polysaccharide, dextran, was unavailable for microbial uptake when complexed with montmorillonite. Similarly, in their study of biological degradation of the pesticide 2,4-D in soils, Ogram and co-workers (1985) proposed that the sorbed phase was protected from biological attack.

Several mechanisms have been implicated in limiting bioavailability in soil-water suspensions, including sorption of the substrate to soil particles (Guerin and Boyd, 1992; Miller and Alexander, 1991), the effect of viscosity of the suspension on mass transport (Verschueren and Visschers, 1988), and intraparticle diffusion (Mihelcic and Luthy, 1991; Rijnaarts *et al*, 1990; Steinberg *et al*, 1987). The following sections will discuss each of these mechanisms in more detail.

2.3.1 Adsorption

Sorption on sediment and soil particles is a significant fate for organic pollutants in aquatic and soil environments. The affinity of a compound to bind to soil surfaces is described by the empirical parameter K_d , usually obtained by fitting adsorption data to linear Freundlich or Langmuir isotherms. The physical interpretation of K_d is that it represents sorption capacity, or the number of adsorption sites available (Weber, 1972). The term sorption has often been used as a general term to describe any association between a compound in solution and a solid surface. Hassett and coworkers (1981) describe sorption as the result of a solution component concentration at an

interface. From a thermodynamic standpoint, sorption of solute molecules can occur when the attraction between the solute and the sorbing surface overcomes attraction of the solvent for the solute, or repulsion by the surface.

Different mechanisms can bring about a sorptive association, and the type of mechanism is important when factors like pH, ionic strength, and dominant cation in the solution phase are considered. For a polar or ionic compound, sorption can be caused by strong short range forces acting between the solute and the surface, such as covalent or hydrogen bonding, or Van der Waals attractive forces (Harter, 1977; Hassett and Banwart, 1989). A nonpolar, nonionic, hydrophobic compound such as naphthalene lacks the polarity or functional groups necessary to create a strong affinity for the sorbent. Sorption of naphthalene in an aqueous solution is therefore more likely to occur as a result of weak solvent-solute attraction.

Many researchers have demonstrated a correlation between hydrophobic organic contaminant sorption and the natural organic carbon content of soils and sediments (Karickhoff *et al*, 1979; Means *et al*, 1980). In whole soils, the organic carbon is believed to provide a hydrophobic environment for the partitioning of nonpolar compounds (Hassett and Banwart, 1989). In such cases, the parameter K_d is often modified to describe sorption solely in terms of percent organic carbon. The modified parameter is $K_{oc} = K_d/f_{oc}$, where f_{oc} is the fractional mass of organic carbon in the sediment or soil (Karickhoff *et al*, 1979). The K_{oc} is considered to be compound specific, and can be approximated from other properties such as water solubility, the experimentally

determined octanol-water partition coefficient (K_{ow}), and molecular structure (Hassett and Banwart, 1989). In low carbon environments, it has been suggested that sorption to mineral surfaces may play an important role (Banerjee *et al*, 1985), although this has not been consistently demonstrated. Stauffer and co-workers (1989) attempted to correlate sorption of nonpolar organic chemicals with various properties of low carbon content aquifer materials (percentage sand, silt, and clay, clay mineral type, specific surface area, cation exchange capacity), but concluded that the sorption coefficient did not correlate with any of the aquifer properties including percent organic carbon.

Adsorption isotherm data is often used as a measure of bioavailability by allowing a prediction of the portion of a contaminant that may be dissolved in the solution phase in a soil-water matrix (Jury *et al*, 1983a; Verschueren and Visschers, 1988). Another approach is to determine the potential for a sorbed contaminant to leach back into the solution phase by obtaining a desorption parameter, K_{des} which represents desorption capacity, or the number of reversible adsorption sites. Assuming that all the desorbed compound is bioavailable, Korde (1991) proposed that K_{des} should give an indication of biodegradation potential. However, Korde's experimental results showed that K_{des} did not correlate well with the biodegradation rate of naphthalene in aqueous suspensions of four widely varying soils. In observing the biodegradation rate of toluene sorbed to soils, Robinson and coworkers (1990) indicated that while most of the toluene desorbed rapidly and was immediately available to bacteria, a small portion exhibited very slow desorption which limited the degradation rate. Pignatello (1989) has

also documented a slow desorbing, residual fraction for PAH's, polychlorinated benzenes, and polychlorobiphenyls in soils and sediments. These studies demonstrate that bioavailability can not always be predicted based on the isotherm coefficient alone.

2.3.2 External Mass Transfer

In film theory, mass transfer into or out of a moving fluid phase occurs by molecular diffusion through a conceptual film of stagnant fluid at the phase boundary. Mass transfer through the film is presumed to be slower by orders of magnitude than it is in the bulk phase and controls the overall rate (Letterman *et al*, 1974). For a neutrally buoyant spherical particle suspended in a turbulent fluid, the coefficient of mass transfer between the particle and the fluid depends on the diffusion coefficient, the particle diameter, the kinematic viscosity of the fluid, and the relative velocity gradient in the fluid surrounding the particle (Sherwood and Brian, 1968). Lawrence and coworkers (1987) state that microorganisms existing at a surface interface are surrounded by a hydrodynamic boundary layer through which nutrients and wastes must diffuse. The boundary layer decreases in size with increasing flow velocity, therefore, depending on mixing rate, conditions within the boundary layer are often diffusion-limited, and nutrients may be at low concentrations. Mass transfer limitations have also been reported for flocculating microbial cultures (Ngian *et al*, 1977).

It is generally assumed that with sufficient mixing, external mass transfer is not a limiting factor in aqueous suspensions. However

sufficient mixing may be difficult to achieve in systems which are characterized by high viscosity. In an examination of viscosity as a factor in bioavailability, Verschueren and Visschers (1988) reported that the quantity of soil has an effect on the viscosity of a soil-water mixture, thus potentially affecting the bioavailability of compounds present in the aqueous phase. Stotzky (1966a) indicated that the addition of 8% montmorillonite (by weight) to an aqueous suspension increased the viscosity to the point that diffusion of oxygen appeared to be impaired. In measuring mass transfer coefficients for particles suspended in agitated tanks, Harriott (1962) observed that the main effect of increased viscosity was a decrease in the molecular diffusivity of the dissolved species, which in turn resulted in a decreased mass transfer coefficient. The addition of particles to a solution has the effect of decreasing the diffusivity of a dissolved compound as the molecules must travel a tortuous path around the suspended particles. Thibodeaux (1979) presents a mathematical expression for an obstructed diffusion path in which the molecular diffusivity is modified to take into account particle shape, volume, and the tortuosity of the diffusion path. The viscosity of a suspension, and its effect on the diffusivity of a dissolved compound, may therefore play an important role in the determination of whether or not a researcher can ignore external mass transfer limitations.

2.3.3 Intra-particle Diffusion

Studies of contaminant fate and transport in porous media have resulted in various models to account for short term, long term and non-

equilibrium behavior (Brusseau, 1991; Jury *et al*, 1983b; Karickhoff and Morris, 1985). Recent studies have shown that hydrophobic compounds such as PAH's and other hydrocarbons exhibit slow desorption kinetics (Pignatello, 1989; Robinson *et al*, 1990). Since its introduction by Wu and Gschwend (1986), the radial diffusion model for sorption kinetics has frequently been adopted to model biodegradation kinetics in soil containing systems (Dhawan *et al*, 1991; Scow and Hutson, 1992; Steinberg *et al*, 1987). In radial diffusion type models, the soil matrix is viewed as a collection of spherical porous aggregates surrounded by a liquid phase. When concentration in the bulk liquid phase is high, molecules of solute diffuse into aggregate micropores and sorb to pore surfaces. As bulk phase concentration decreases, desorption from pore surfaces is rapid, but transport of the solute is limited by intraparticle diffusion. These models assume no external mass transfer limitations.

Scow and Hutson (1992) presented a diffusion-sorption-biodegradation (DSB) model which combined diffusion as described by Fick's second law, sorption described by a linear isotherm, and first-order biodegradation kinetics with the Wu and Gschwend (1986) conceptual model of a spherical, porous soil aggregate. The DSB model was used to perform a sensitivity analysis on the relative contributions of diffusivity, adsorption coefficient, and biodegradation rate on persistence of a compound in soil. Results indicated that intraparticle diffusion plays a less significant role when small radius aggregates are present, or at high ratios of solution outside to solution inside aggregates. In the same study, it was also reported that intraparticle diffusion may be less significant in saturated, slurried, or poorly

aggregated soils. These observations suggest that it may be possible to rule out intraparticle diffusion as a biodegradation rate-limiting mechanism in aqueous suspensions.

2.3.4 Clay/Microbe Interactions

In soils, bacteria as well as surfaces are predominantly negatively charged (Santoro and Stotzky, 1968; van Loosdrecht *et al*, 1990). In bacteria, surface charge results primarily from ionic changes in cell wall components. The like-charged bacterial and particle surfaces will approach each other when van der Waals attractive forces are sufficient to overcome electrostatic repulsion. Repulsion energy is high at small interparticle distances, and decreases with increasing particle separation to a point where attractive forces begin to dominate. The separation distance required for attraction to occur is highly dependent on factors that influence electrostatic charges such as pH, ionic strength, and valency of the dominant cations in the system. An excellent summary of the effect of separation distance on bacteria-particle interaction can be found in van Loosdrecht *et al* (1989).

Marshall (1980) described three types of interactions between bacteria and soil particles: (1) adsorption of bacteria on the surface of larger soil particles, (2) aggregation of bacteria and soil particles of similar size, and (3) adsorption of colloidal particles at the bacterial surface. Such bacteria-particle interactions were further defined as adhesion or attachment, either reversible, or permanent. Descriptions of bacteria-particle interactions seldom contain more details than provided by this conceptual view. Due to the complexity of the soil

habitat, studies concerning these interactions have usually been limited to an examination of particular soil fractions and organisms under highly controlled conditions. As a consequence of reducing the variables to a manageable number, results are often inconclusive, and data from different studies can seldom be compared on a like basis.

There has been much speculation regarding the effects of adhesion on microbial metabolism. Organisms existing at a solid interface may encounter conditions that are significantly different from those faced by suspended cells in a nearby bulk solution phase. Environmental factors that may be influenced by particle surfaces include pH, availability of oxygen, proximity of other cells, or availability of nutrients which can be concentrated at particle surfaces (Stotzky, 1985). Attachment may alter cell mobility, or decrease cell surface area available for nutrient uptake (Jeffrey and Paul, 1986). There may also be increased competition for oxygen and nutrients among cells grouped together at an interface.

Studies concerning the effects of clay minerals on bacterial growth and substrate utilization have been confounded by complex interactions between bacteria and clay, bacteria and substrate, and substrate and clay (Marshman and Marshall, 1981a). Examples from the literature show widely varying results depending on both type and amount of clay mineral, substrate, and organisms. Inhibition is usually proposed to be the result of a clay-substrate interaction, such as adsorption, complexation, or ion exchange (Marshman and Marshall, 1981b; Miller and Alexander, 1991; Olness and Clapp, 1972). In contrast, enhancement is most often attributed to protective effects of clays on

the organisms (from dessication, pH changes, or protozoan grazing to name a few) (Kunc and Stotzky, 1974; Marshall, 1968; Stotzky and Rem, 1966; van Loosdrecht *et al*, 1990). Stated in another way, modification of the physicochemical characteristics of the microbial habitat by clays may be described as indirect effects, and clay-microbe interactions (such as adhesion) as direct effects.

Kunc and Stotzky (1974) studied the effect of different clay minerals on the activity of soil microorganisms. A variety of organic substrates was added to soils that had been ammended with montmorillonite, kaolinite, or mica-vermiculite. The 100g soil-clay mixtures were adjusted to 1/3 bar water content and continuously aerated. Microbial activity was measured periodically by CO₂ evolution and O₂ consumption. Results indicated that the addition of clay minerals could enhance, inhibit, or have no effect on the rate and/or the extent of metabolism of various organic substrates.

In another study, Marshman and Marshall (1981b) examined growth of soil microorganisms on various proteins in the presence of montmorillonite and kaolinite. It was observed that the rate and extent of protein utilization was dependent on the relative concentrations of protein and clay in the medium, with each combination of organisms, minerals and proteins exhibiting quantitative differences. Three different protein-to-clay ratios were found to induce three different growth curves, with no effect at high protein-to-clay ratios, reduced growth at intermediate protein-to-clay ratios, and no protein hydrolysis at low protein-to-clay ratios. Those same ratios did not appear to be correlated with adsorption of the protein on the clay. One conclusion

was that sorbed protein was conditionally bioavailable; it was proposed that the clays provided two types of binding sites for the protein molecules, with only one of the sites being susceptible to microbial attack. Another possibility that was not suggested is that the clays had a direct effect on the microorganisms unrelated to the protein-clay interaction.

Often, as in the Scow and Hutson (1992) DSB model mentioned previously, the disappearance of substrate is modeled mathematically by combining bacterial growth kinetics with terms to account for the unavailability of a portion of the substrate due to alternate fates such as volatilization, sorption or slow diffusion. Mihelcic and Luthy (1991) coupled Michaelis-Menten kinetics with an intraaggregate radial diffusion model to describe their observations of microbial degradation of naphthalene in soil-water suspensions, using the assumption that sorbed solute was inaccessible to microorganisms. Miller and Alexander (1991) observed that mineralization of benzylamine in montmorillonite-water suspensions was slower than in the absence of the clay. Again, the assumption was made that bioavailability of benzylamine was decreased due to adsorption onto the clay, and a model incorporating first-order biodegradation kinetics and an adsorption coefficient for benzylamine was developed that fit the data satisfactorily. In some of the experiments by Kunc and Stotzky (1974) however, montmorillonite was found to increase the rate of mineralization of several amino acids and aldehydes, with no measure made of adsorption of substrate. It becomes clear rather quickly that results are highly experiment specific, differences in experimental methods make comparisons

problematic, and general conclusions about clay-microbe systems are almost impossible to make.

The wide array of experimental conditions is prohibitive to the consistent use of any particular model. As a result, variations on models have grown to such an extent that virtually every study boasts its own unique version. Scow and coworkers (1986), in measuring mineralization of phenol and aniline in soil, compared the fit of Monod-derived models (first-order, integrated Monod, logistic, logarithmic and zero-order), as well as three-half-order and two compartment models. Only the two compartment model could provide a reasonable fit, leading to the subsequent study in which diffusion and sorption terms were added to come up with the DSB radial diffusion type of model (Scow and Hutson, 1992).

Occasionally, experimental results defy even the most astute manipulation of data. In a study by Korde (1991), the extent of naphthalene mineralization was found to correlate almost perfectly and inversely with the percent clay in four different soils whereas correlation with the presence of other soil constituents and with sorption coefficients was weak to non-existent. The surprising implication in this last study was that biodegradation of naphthalene did not conform strongly to previous observations whereby increased sorption indicated decreased bioavailability.

Although the most frequent interpretations of clay effects on microbial activity are concerned with clay-substrate interactions, certain researchers have suggested that clay minerals may have a direct effect on bacteria. Ogram and co-workers (1985) found no

measurable sorption of either bacteria or 2,4-D to montmorillonite, but suggested that small clay particles may have sorbed to the bacteria inhibiting 2,4-D from diffusing past the clay barrier and into the cell. Marshall (1969) observed adsorption of montmorillonite clay on rhizobia by electron microscopy. The electron micrographs confirmed that in a suspension of colloidal clay and bacteria, the outer surface of the bacteria was covered by an envelope of adsorbed clay particles. Similar adsorbed quantities were observed for two different types of clay minerals, leading Marshall to propose that the surface characteristics of the organisms rather than specific mineralogical properties were the most important factors determining the interaction. In the same study, Marshall found that the electrophoretic mobilities of four rhizobia isolates, when mixed with clay were the same as the mobility of the clay alone, whereas in the absence of clay, the four isolates exhibited quite different mobilities. This alteration in the microbial surface characteristics was taken to be a further confirmation of the presence of a clay envelope surrounding the organisms.

2.4 Biodegradation Assays

2.4.1 Biodegradation by Suspended Cells

In both continuous flow and batch laboratory studies to determine treatability of a contaminated soil by bioremediation, the microbial population is commonly added as a suspension. However, there appears to be little consistency in the manner in which cell

cultures are prepared prior to use. Table 1 presents a selection from the literature of several different methods of cell culture and preparation. Growth media have not been identified in the table as they are assumed to be species specific. Except for two instances when a specific growth phase was desired (stationary phase or late log phase), the reasons for choosing a particular length of time for cell growth were not given. While these examples have not been singled out for any particular fault in methodology, the list serves to illustrate the difficulty in making comparisons among different researchers, or even among different studies performed by the same researcher.

The varying characteristics of microbial populations at different growth stages are well documented in basic texts on microbiology such as Schlegel (1985). Important parameters of a bacterial culture include yield (the difference between initial and maximum bacterial mass), growth rate, and duration of the lag phase. Any and all of these parameters can have an effect on experimental results, and lack of understanding about the age-related characteristics of the culture being used may lead to erroneous conclusions.

2.4.2 Biodegradation by Immobilized Cells

Immobilization of bacterial cells within an insoluble matrix has been utilized as a means of protecting the cells against an inhibitory environmental condition such as a toxic substrate concentration (Bettmann and Rehm, 1984). Use of immobilized cells is also advantageous in bioreactors for preventing washout and maintaining

Table 1. Variations in cell culture methods.

Reference	Cell culture method
Marshall, 1968	•Grown until reaching stationary phase (anywhere from 3-12 days) •Washed twice and resuspended in 0.1M NaCl
Marshman and Marshall, 1981b	•Grown for 18 hours •Resuspended in water
Miller and Alexander, 1991	•Grown for unspecified length of time •Washed once with phosphate buffer •Resuspended in salts solution
Ogram <i>et al</i> , 1985	•Grown until reaching late log phase (4 to 5 days) •Washed once and resuspended in phosphate buffer
Robinson <i>et al</i> , 1990	•Grown for unspecified length of time •Washed 3 times and resuspended in salts solution
Santoro and Stotzky, 1968	•Grown for unspecified length of time •Washed at least 3 times with water
Stotzky, 1966a	•Grown for 17 to 24 hours •In some cases, cells were washed at least 3 times with water
Stotzky and Rem, 1966	•Continuous culture maintained which was transferred to fresh medium every 18-48 hours •Washed 3 times with water
Vandenhove <i>et al</i> , 1991	•Grown for 16 hours •Washed once and resuspended in distilled water

high cell density. There are several methods for immobilizing live cells, including covalent coupling, adsorption, confinement in a liquid-liquid emulsion, entrapment within a semipermeable membrane, and entrapment within a three-dimensional polymer network (O'Reilly and Crawford, 1989). One such three-dimensional matrix is constructed of alginate, a copolymer of D-mannuronic and L-guluronic acid cross-linked by calcium ions (Yang and Wang, 1990). Alginate forms solid, spherical beads; oxygen and nutrients must diffuse through the outer matrix layers to reach bacterial cells deeper within the beads. Therefore, diffusion of oxygen and substrate through the immobilization matrix may become limiting. O'Reilly and Crawford (1989) suggested oxygen diffusion as limiting the rate of *p*-cresol degradation by alginate-immobilized-cells in both batch and continuous flow systems. The use of immobilized cells would appear to be an ideal method for studying clay-microbe interactions by eliminating direct cell-particle contact while maintaining the physicochemical properties of the suspension, however no such studies were found in the literature.

2.4.3 Mineralization

A common method of quantifying microbial activity is to measure respiration. Both O₂ uptake and CO₂ production have been used extensively in this respect (Kunc and Stotzky, 1974; Olness and Clapp, 1972; Stotzky, 1966a and b; Stotzky and Rem, 1966). While measurements of total O₂ uptake and CO₂ production limit the data to an overall indication of microbial respiration, the use of a ¹⁴C-labeled substrate allows a more specific determination of the quantity of compound that

has been completely mineralized to $^{14}\text{CO}_2$. Use of a radiolabeled compound also allows the researcher to calculate a mass balance for purposes of identifying different fates in the experimental system. Radiolabeled naphthalene has been used successfully in several mineralization studies (Burlage *et al*, 1990; DiGrazia, 1991; Korde, 1991), establishing a solid groundwork for future research.

2.4.4 Bioluminescence

A recent advance in the detection of naphthalene metabolism is the construction of bioluminescent reporter strains of *Pseudomonas fluorescens*. These organisms contain a specific catabolic plasmid that contains genetic information for both the production of light and the naphthalene degradative pathway. The light response is induced when the organisms are exposed to aqueous- or vapor-phase naphthalene, or when grown in the presence of salicylate. Bioluminescence assays are unique in their sensitivity, specificity, and rapid response times. In addition, direct measurements of cell metabolic activity can be made without disruption of the experimental environment. However, difficulties in the interpretation of light intensity mean that much of the data will be qualitative in nature. An investigation by King and coworkers (1990) demonstrated a relationship between light production and rate of naphthalene degradation in aqueous environments by a *Pseudomonas fluorescens* strain containing the pUTK21 bioluminescent reporter plasmid for naphthalene catabolism. A similar relationship could not be determined in soil-containing systems either in this or in a subsequent study (DiGrazia, 1991). In both studies, the difficulties with

the soil slurries were attributed to quenching of the light and interference in the detection system by the soil matrix. Therefore, soil slurry data were used only as a qualitative indication of biodegradative activity.

3.0 APPROACH

A key to understanding the role of clays in microbial degradation is the ability to identify indirect (clay-substrate) from direct (clay-microbe) interactions. With this in mind, the starting point for this study was an abiotic characterization of the substrate-clay interaction. The biological experiments were then designed from the standpoint of observing the effects of various clay concentrations on the rate of microbial activity in the presence of naphthalene, and testing for possible rate limiting mechanisms.

3.1 Choice of Clays

One of the goals of this study was the ability to directly compare results with those obtained by other researchers. One of the first steps toward achieving this goal was the decision to use fully characterized clays from the Clay Minerals Society Source Clays Repository. These clays have been used extensively in studies that span several disciplines, and information about their characteristics and behavior is easily obtained. Foreknowledge of the properties of the individual clays is extremely useful in providing a check on the accuracy of experimental methods. Additionally, use of these commercially available clays allows this particular research to be more easily duplicated and expanded upon without loss of continuity. It was decided to begin the abiotic characterization with six different clays exhibiting a wide range of physicochemical properties as shown in Table 2. Of particular interest were the cation exchange capacity (CEC)

Table 2. Physical and chemical properties of six source clays.

Clay	Mineral Structure	Cation Exchange Capacity (CEC) (cmol kg ⁻¹)	N ₂ Surface Area (m ² g ⁻¹)
KGa-1 Kaolinite	1:1 layer silicate, well-crystallized	2.0	10.05 ± 0.02
KGa-2 Kaolinite	1:1 layer silicate, poorly crystallized	3.3	23.50 ± 0.06
PFI-1 Attapulgite (Polygorskite)	crystallizes in the form of long needles	19.5	136.35 ± 0.31
SAz-1 Montmorillonite Arizona	2:1 layer silicate expanding smectite Al ⁺⁺⁺ predominant	120.0	97.42 ± 0.58
SHCa-1 Hectorite California	2:1 layer silicate expanding smectite Mg ⁺⁺ predominant	43.9	63.19 ± 0.50
STx-1 Montmorillonite Texas	2:1 layer silicate expanding smectite Al ⁺⁺⁺ predominant	84.4	83.79 ± 0.22

and surface area. The CEC was expected to influence the hydrophobicity of the clay surfaces by retention of hydrated cations, while the surface area was anticipated to directly reflect the number of sorptive sites. The design of the subsequent biological experiments would then incorporate those properties which would appear most likely to affect the bioavailability of naphthalene.

3.2 Choice of Assays

One objective of the biological experiments was to verify the findings made by Korde (1991) correlating increased clay concentration with decreased extent of naphthalene mineralization. Therefore, the

methods and materials for the mineralization assay were chosen to be similar to those used in that study. The assay had the advantage of providing a quantifiable measure of naphthalene mineralization through the use of radiolabeled compound, however, it was quite costly to perform in terms of labor and materials, making its repeated use somewhat prohibitive. As an alternative, the bioluminescence assay had recently become an option for the detection of naphthalene degradation in aqueous and soil-containing systems. Bioluminescence detection had the advantage of being rapid, noninvasive, and inexpensive (after expending the initial cost for the detection instrumentation). Use of bioluminescence as an assay also allowed the observation of instantaneous rather than cumulative effects. Additionally, it was seen to be particularly useful in experiments designed to measure mass transfer limitations by allowing a wide range of mixing speeds without concern of upsetting the CO₂ trapping device within a mineralization vial. To make the transition from mineralization to bioluminescence, a correlating experiment was required. It was also necessary to determine whether the presence of the clay interfered with the efficiency of light detection as was observed by DiGrazia (1991). This created the need for conducting several experiments to identify any light-quenching or shading effects.

3.3 Choice of Microorganisms

The choice of microorganism was a direct result of the choice of assay. To provide a tie-in with the work done by Korde, the strain 5R was chosen for the mineralization experiments. As 5R was not a light-

producing organism, the transition to bioluminescence required a switch in strain. The related strain HK44 was chosen as its growth characteristics and light-producing ability had been fairly well identified by other researchers (Burlage *et al*, 1990; DiGrazia, 1991, DiGrazia *et al*, 1990). As a check on the light detection method, some experimentation was done with strain 5RL which produced bioluminescence similar to HK44 but in response to a different substrate. Pure cultures were used in all cases both for comparative purposes, and to limit experimental variables.

3.4 Experimental Approach

As mentioned previously, this study began with the abiotic characterization of the naphthalene-clay interaction. The adsorption isotherm was the chosen vehicle for this characterization, but rather than limiting the examination to a single set of test conditions, it was decided to build an experimental matrix by varying clay type, pH, ionic strength, and dominant cation. These variables were selected for the effects that they would have on the degree of hydration of the clay surfaces, which would in turn affect the extent to which hydrophobic naphthalene molecules would approach those surfaces. The size of the matrix was reduced statistically to a manageable number of experiments. The statistical reduction used an iterative process to randomize the experimental variables and select the number of replicates which would represent the entire matrix at a chosen efficiency of 85% or greater (Evolutionary Software, Inc., 1991).

Following the abiotic characterization, two clays were chosen for the mineralization experiments, the results of which could be compared to those observed by Korde (1991). The KGa-2 kaolinite was selected for its low specific surface area, and the SAz-1 montmorillonite was selected for exhibiting the least sorptive capacity for naphthalene at neutral pH. The transition was then made to bioluminescence experiments, with experiments designed to identify whether a particular rate-limiting mechanism was a factor. As the abiotic experiments had indicated that adsorption was not significant, it was eliminated as the subject of further testing. External mass transfer limitations were tested for by observing the effects of various mixing speeds on light production. To determine whether intraparticle diffusion was significant within the time frame of the experiment, light production was measured in samples in which the clay had been exposed to naphthalene overnight and in samples in which naphthalene was added immediately before addition of the microbes. Direct clay-microbe contact was tested for by the addition of dispersing and flocculating agents, substitution of fine sand for the clay, and by eliminating the possibility for direct contact through the use of cells immobilized in alginate. The experiments as designed led to conclusions by the process of elimination, demonstrating that even in a highly simplified and artificial system, the absolute identification of a causative mechanism is extremely challenging.

4.0 EXPERIMENTAL

4.1 Abiotic Studies

A well-crystallized kaolinite (KGa-1), a poorly crystallized kaolinite (KGa-2), Florida attapulgite (PFl-1), Arizona montmorillonite (SAz-1), California hectorite (SHCa-1) and Texas montmorillonite (STx-1) were obtained from the Clay Minerals Society Source Clays Repository, Columbia, MO. Physical and chemical properties of the six clays are provided in Table 2 in Section 3.1. For the abiotic characterization, an experimental matrix was developed which consisted of six different clays, three pH conditions, two variations in ionic strength, and three alternatives for dominant cation. An iterative statistical program was used which randomized the 108 combinations of the four experimental design factors, and selected 24 whose results would represent those of the complete matrix at an efficiency level of 89 to 100% (Evolutionary Software, Inc., 1991). The reduced matrix shown in Table 3, formed the basis for the procedures described below.

4.1.1 Clay Preparation

Preparation of clay samples prior to adsorption isotherm experiments included removal of carbonates and soluble salts, removal of organic matter, and removal of iron oxides, all as described in Gee and Bauder (1986). Clay samples were then saturated with the appropriate cation selected from the experimental matrix. All clays were pretreated in 20 g batches using 250 mL polypropylene centrifuge bottles. All centrifugation steps were carried out at 3000 rpm for 15 min at 25°C. Potassium, calcium, and aluminum saturated batches were made up from

Table 3. Matrix showing conditions for adsorption isotherm experiments 1 - 24.

	KGa-1	KGa-2	PFl-1	SAz-1	SHCa-1	STx-1
pH						
4.0	1,3	7,8	9	13,15		22,23
5.5	2	6	10,12	14	19,20	21
7.0	4	5	11	16	17,18	24
Ionic Strength						
0.01	1,4	6,7,8	10,11	13,16	17,19	21,22
0.1	2,3	5	9,12	14,15	18,20	23,24
Cation						
K ⁺	1	5	9	13,14	17	21
Ca ⁺⁺	2	6	10,11	15	18	22,23
Al ⁺⁺⁺	3,4	7,8	12	16	19,20	24

Numbers within the matrix correspond to experiment number.

Clay identification key:

KGa-1	-Kaolinite, well crystallized
KGa-2	-Kaolinite, poorly crystallized
PFl-1	-Attapulgite, Florida
SAz-1	-Montmorillonite, Arizona (Cheto)
SHCa-1	-Hectorite, California
STx-1	-Montmorillonite, Texas

each of the six clays. Cation saturation was accomplished by five sequential washings in 1 M KCl for potassium saturated clays, 0.5 M $\text{Ca}(\text{OAc})_2$ for calcium saturated clays, and 0.33 M AlCl_3 for aluminum saturated clays. Each washing consisted of vigorous shaking by hand for 5 - 10 minutes to completely disperse the clay, followed by centrifugation. The supernatant solution was then poured off and discarded, and replaced with fresh washing solution. Following cation saturation, clay samples were rinsed repeatedly with distilled, deionized water for a maximum of seven rinsings, or until no chlorides were present in the supernatant as indicated by precipitation with 1 M AgNO_3 . After the final water rinse, centrifugation and pouring off of the supernatant, the samples were freeze dried.

4.1.2 Specific Surface Area and Particle Size Analyses

Specific surface area was measured by low-temperature nitrogen adsorption (ASAP 2000, Micromeritics, Norcross, GA). This method utilizes the Brunauer, Emmett and Teller (BET) equation for calculating specific surface area as a function of adsorbed volume of N_2 at a known pressure, the volume of N_2 required to form a monolayer over the sorbent surface, and the cross-sectional area of the N_2 molecule.

Although the saturating cation can affect the interlayer surface area of expanding clay minerals (Bohn *et al*, 1985), the external surface area, which is measured by the nitrogen-BET method, is not expected to vary with the cation. Thus, surface area measurements were made only on the six different potassium-saturated clays.

The approximate particle size range for the clay mineral fraction, given as 0.2 - 2 μm , was sufficient information for most of the clays used in this study. The exception was the montmorillonite (SAz-1) used in the majority of the biological experiments. A more detailed particle size analysis of pretreated and untreated SAz-1 was performed by Coulter Counter using the Isotone dispersing agent, and a 70 μm and 50 μm aperture respectively.

4.1.3 Adsorption Isotherms

Solutions used to perform the naphthalene adsorption isotherms were made up to the desired ionic strength using distilled, deionized water and a salt of the saturating cation, either KCl, CaCl_2 , or AlCl_3 . Hydrogen ion activity was adjusted to the desired value using HCl or NaOH as needed. Naphthalene crystals were then added to each salt solution to produce a concentration of 25 mg L^{-1} naphthalene. The solutions were closed tightly and stirred until the crystals were dissolved. Finally, $1\text{-}^{14}\text{C}$ -naphthalene (Sigma, St. Louis, MO) was added to give approximately 20,000 dpm mL^{-1} .

Adsorption experiments were performed using duplicate samples with a 1:5 clay:solution ratio (1 g clay with 5 mL solution). The 5 mL solution contained naphthalene-free electrolyte (with cation matching the dominant clay cation) plus naphthalene-containing electrolyte in six different ratios to provide initial concentrations of 0.0, 5.0, 10.0, 15.0, 20.0, and 25.0 $\mu\text{g mL}^{-1}$ naphthalene. Mercuric chloride (0.005g per gram of clay) was added to each tube to inhibit biological activity. Test tubes were glass with Teflon-lined screw caps. The test tubes were

tightly capped, vortexed briefly, enclosed in a Styrofoam box and equilibrated on a reciprocating shaker at 150 cps. After 24 hours, the suspensions were centrifuged at 3,000 rpm for 15 minutes. A 0.5 mL sample of each supernatant was immediately placed into 10 mL aqueous scintillation cocktail (Beckman Ready-Safe) and counted on a Beckman scintillation counter. After direct quantification of solution phase naphthalene, the adsorbed quantity was calculated by difference.

4.2 Biological studies

4.2.1 Media

Microbial cultures were grown on liquid and/or agar yeast extract, peptone, glucose (YEPG) media (Korde, 1991). Three different formulations of mineral salts solutions were used in the mineralization and bioluminescence experiments. Basal salts medium (BSM) was prepared by adding (g L^{-1}): NaNO_3 , 4.0; KH_2PO_4 , 1.5; FeCl_3 , 0.005; MgSO_4 , 0.2; CaCl_2 , 0.01; Na_2HPO_4 , 0.5; and adjusting to pH 7.0 with HCl or NaOH before autoclaving. Modified basal salts medium (MBSM) contained (g L^{-1}): NaNO_3 , 4.0; KH_2PO_4 , 0.005; FeCl_3 , 0.005; MgSO_4 , 0.2; CaCl_2 , 0.01; KCl, 1.75; adjusted to pH 7.0 as for BSM, and buffered with 0.05 M Tris(hydroxymethyl)aminomethane (TRIS). Soil solution medium (SSM), based on Angle *et al*, (1991) contained (g L^{-1}): FeCl_3 , 0.005; MgSO_4 , 0.2; CaCl_2 , 0.4; Na_2HPO_4 , 0.001; NH_4NO_3 , 0.2; KCl, 0.04; NaNO_3 , 0.2; adjusted to pH 7.0 and buffered with 0.05 M TRIS.

4.2.2 Microbial Strains

Three different strains of *Pseudomonas fluorescens* were used in this study. Strain 5R, isolated from a Manufactured Gas Plant (MGP) soil, contains the naphthalene catabolic genes (King *et al*, 1990). 5R was used to produce strain 5RL, a bioluminescent construct containing the plasmid pUTK21. Due to the insertion point of the plasmid, 5RL does not completely mineralize naphthalene, but accumulates salicylate. A separate MGP isolate was used to produce HK44, a bioluminescent reporter strain also containing pUTK21. HK44 has similar light-producing characteristics to 5RL, and is able to completely mineralize naphthalene. All three strains were initially maintained on one-quarter strength YEPG agar plates from which liquid media were inoculated (Growth Procedure I). Later, frozen stock vials of HK44 grown on full-strength YEPG were prepared by mixing the inoculum culture with a few drops of glycerol (Growth Procedure II). Frozen stocks were maintained at -90°C.

The plasmidless *Pseudomonas putida* strain PB2440 has no naphthalene degradative ability, and was used in control replicates during the mineralization assay. Growth Procedure I was used to maintain and prepare PB2440.

4.2.3 Suspended Cells

This section contains a description of the two methods used to produce cultures of suspended cells for mineralization and bioluminescence assays.

4.2.3.1 Growth Procedure I

Full-strength YEPG medium was inoculated directly from plate cultures and placed in a shaking growth chamber (27°C, 100 rpm) for 12 - 16 hours. A 25 mL volume of cell suspension was removed to a 50 mL polypropylene centrifuge tube and centrifuged at 10,000 rpm for 15 min at 25°C. The supernatant solution was decanted and the pellet was re-suspended in 25 mL BSM. The cells were re-centrifuged and resuspended in BSM, centrifuged one last time, then resuspended in 5 mL BSM to give a final cell concentration of approximately 10^9 cells mL⁻¹.

4.2.3.2 Growth Procedure II

A vial of frozen stock was thawed, added to full-strength YEPG medium, and grown for 12 - 16 hours as in Growth Procedure I. A 6 mL volume of culture was transferred to fresh YEPG and placed in a shaking water bath at 26°C. The optical density of the culture at 546 nm was measured every half hour, and the value was plotted versus time on semi-logarithmic graph paper. The linearity of the plot was used to verify exponential growth. Cells were harvested at an optical density of 0.3 (O.D.₅₄₆), corresponding to approximately 3×10^8 cells mL⁻¹. Cells prepared by this growth procedure were added directly to experimental vials along with a volume of the YEPG growth medium.

4.2.4 Immobilized Cells

Immobilization of cells began with an overnight culture grown in YEPG. Two 35 mL volumes of suspended cells were removed from the

culture and centrifuged at 8,000 rpm for 10 minutes at 25°C. Each pellet was resuspended in 10 mL 0.9% NaCl solution, and the optical density (O.D.₅₄₆) was determined. One volume of cell suspension was then mixed gently with two volumes of alginate solution (2.8 g low viscosity alginate (Sigma, St. Louis, MO) dissolved in 100 mL of 0.9% NaCl). A 20 mL volume of the cell-alginate mixture was added dropwise through a 26 gage needle into 100 mL slowly stirring 0.1 M CaCl₂ solution. The newly-formed beads were left stirring in the CaCl₂ solution for 30 - 45 minutes for hardening. Finally, the beads were collected for use by straining through a cheese cloth.

4.2.5 Mineralization Assays

The mineralization assay was prepared by setting up five replicates of 40 mL, Teflon-septum, screw cap vials (I-Chem) for three clay conditions (1 g KGa-2 kaolinite, 4 g KGa-2 kaolinite and 1 g SAz-1 montmorillonite) and 13 time points (hours 0 - 12). Both clays were pretreated as described in Section 4.1.1, and saturated with potassium. Six control vials were also prepared for each clay condition. A 5 mL volume of BSM containing 25 mg L⁻¹ naphthalene spiked with 10,000 - 20,000 dpm mL⁻¹ ¹⁴C-naphthalene was added to each vial. The vials were mixed well and allowed to equilibrate for 24 hours.

An open 8 mL glass vial containing 0.5 mL 0.4 N NaOH was placed upright into each 40 mL sample vial to serve as a CO₂ trap. A 100 µL volume of strain 5R cell suspension prepared by Growth Procedure I was then added to three of the five replicates, and 100 µL of strain

PB2440 cell suspension, also prepared by Growth Procedure 1, was added to the remaining two replicates. No cells were added to the six control vials for each clay condition. The time at which the microorganisms were added was considered to be Time 0. Plate counts to determine beginning microbial numbers were made from the remaining cell suspension.

Vials were placed in closed boxes, and incubated in a shaking growth chamber (27°C, 100 rpm). At one hour intervals (including Time 0), activity was terminated in the set of vials for that time point by injecting 0.5 mL 2 M H₂SO₄ through the septum. One set of control vials was acidified at Time 0, and the second set at the last time point. After acidification, the mineralization vials were shaken for another hour, after which 4 mL hexane:isopropanol (4:1) was injected, followed by one half hour of shaking. The NaOH was then removed from the CO₂ trap vial by tipping the contents into 1 mL distilled, deionized water and 10 mL ReadySafe scintillation cocktail. The CO₂ trap vial was removed and discarded, and the mineralization vial was resealed, vortexed briefly, and shaken for another hour. A 0.2 mL sample of the hexane phase was placed into 10 mL Dupont Econofluor scintillation cocktail, and the remaining hexane was removed by pipette and discarded. The clay was allowed to settle, and a 0.5 sample was removed from the remaining polar phase and placed into 10 mL ReadySafe. All samples were counted using a Beckman scintillation counter.

For the KGa-2 samples, the assumption was made that 100% of the added naphthalene could be accounted for by summing the amounts recovered in the CO₂ trap, hexane phase, and polar phase. For the SAz-1

samples, an additional sample oxidation step was performed to determine the combined efficiencies of the CO₂ trap, hexane and water extractions. The SAz-1 samples were vortexed and the contents were poured into aluminum weigh pans, and allowed to air dry in a fume hood. After drying, each clay sample was combusted for 4 min in a Harvey Biological Material Oxidizer (model OX-500, R.J. Harvey Instrument Corp., Hillsdale, NJ). The combustion off-gas was collected in 15 mL Harvey Carbon 14 scintillation cocktail for quantification.

4.2.6 Bioluminescence Assays

Due to its low sorptive capacity for naphthalene at neutral pH (determined experimentally, and discussed in Sections 5.2 and 5.3), the clay used in all bioluminescence assays was SAz-1 montmorillonite. Assays were conducted at room temperature (21 - 23°C).

4.2.6.1 Light-Detection Apparatus

Bioluminescence was detected with the use of an Oriel (Stratford, CT) digital display (model 7070) with a photomultiplier tube (model 77340) connected to a flexible liquid light pipe and collimating beam probe (King *et al*, 1990). The probe was inserted through a hole in the side of a hinged box, so that it butted up against the side of a sample vial which was set in an upright position in a Styrofoam holder within the box (Figure 1). The box was opened for placement of the sample vial, then closed tightly for measurement. In between measurements, samples were shaken continuously on an orbital shaker at 100 rpm unless otherwise indicated.

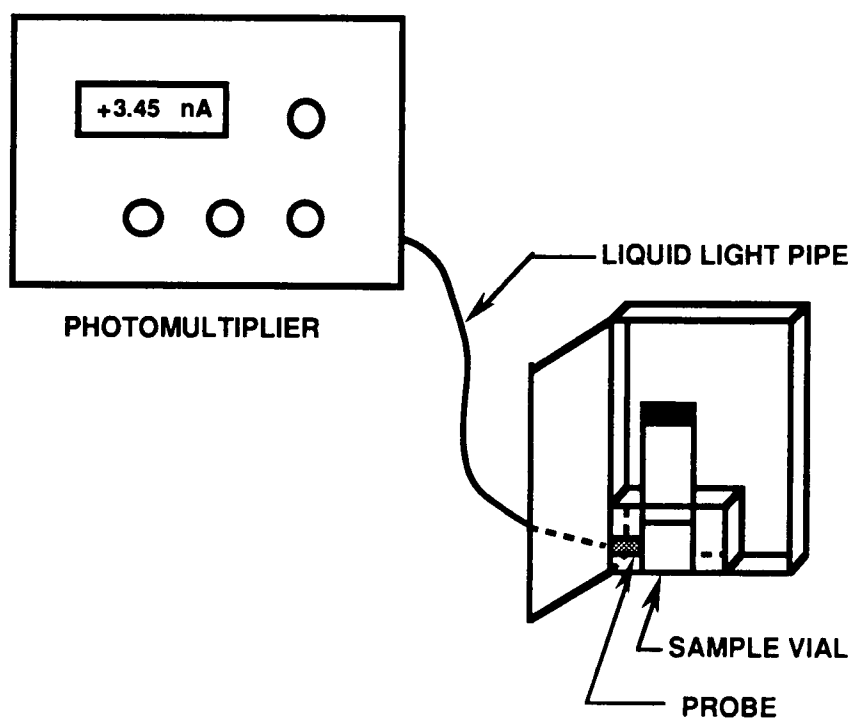


Figure 1. Apparatus for bioluminescence assays

4.2.6.2 Light Response Characterization

Two separate experiments were performed to characterize the light response under different conditions. Clay quantities (g) used for both experiments were 0.0, 0.005, 0.05, 0.1, 0.2 and 0.4. These six clay quantities constituted one set of vials. Sample vials were 40 mL Teflon-septum, screw cap vials (I-Chem). For the first experiment, 5 mL full-strength YEPG containing 0.5 g L^{-1} sodium salicylate was added to one set of vials, and 5 mL one quarter strength YEPG, also containing 0.5 g L^{-1} sodium salicylate was added to a second set. The YEPG media was selected for the purpose of observing the light response in the presence of abundant nutrients. No replicates were made. Each vial received approximately 3×10^9 cells of strain 5RL prepared by Growth Procedure I. In the second experiment, 3 mL MBSM containing 3 mg L^{-1} naphthalene was added to one set of triplicate vials, mixed well, and allowed to equilibrate for 24 hours. The 0.2 g clay condition was not included in this experiment. Another set of triplicate vials containing no clay received 3 mL MBSM with five different naphthalene concentrations (0.0, 0.75, 1.5, 3.0, and 6.0 mg L^{-1}). Each vial received 2 mL of YEPG containing approximately 6×10^8 cells of strain HK44 prepared by Growth Procedure II. Bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.3 Correlation of Bioluminescence with Mineralization

The experiment to correlate bioluminescence with mineralization was conducted using 25 mL rubber-capped Kontes mineralization flasks with sidearm and suspended CO_2 trap. Triplicate flasks of three clay

quantities (0.0 g, 0.2 g, and 2.0 g) were set up. A 9 mL volume of BSM containing 3 mg L⁻¹ naphthalene and spiked with 7,500 dpm mL⁻¹ ¹⁴C-naphthalene was added to each vial. The vials were mixed well and allowed to equilibrate for 24 hours. To begin the assay, 0.5 mL 0.4 N NaOH was placed into each of the suspended CO₂ traps. Each vial received 1 mL of YEPG containing approximately 3x10⁸ cells of strain HK44 prepared by Growth Procedure II. Bioluminescence was measured as described in Section 4.2.6.1 above. One set of flasks was acidified with 0.5 mL 2 M H₂SO₄ at the end of each hour for three hours. At least one hour after acidification of the sample, the NaOH was removed from the CO₂ trap vial and added to 1 mL distilled, deionized water, and 10 mL ReadySafe scintillation cocktail. The clay was allowed to settle, and a 0.5 mL sample of the liquid phase was also removed to 10 mL ReadySafe.

4.2.6.4 Test for Change in Cell Numbers

Two separate experiments were performed to determine whether any changes in cell numbers occurred over the course of a typical bioluminescence assay. The first experiment had two concurrent activities - light detection and population enumeration. Triplicate samples of six clay quantities (0.0 g, 0.005 g, 0.05 g, 0.1 g, 0.2 g and 0.4 g) were set up for light detection. Additionally, three sets of duplicate samples of 0.0 g, 0.05 g, 0.1 g, and 0.4 g clay quantities were set up for population counts. Sample vials were 40 mL Teflon-septum, screw cap vials (I-Chem). A 5 mL volume of MBSM containing 25 mg L⁻¹ naphthalene was added to each vial. The vials were mixed well and allowed to equilibrate for 24 hours. Each vial received approximately

10^9 cells of strain HK44 prepared by Growth Procedure I.

Bioluminescence was measured as described in Section 4.2.6.1 above.

One set of population count vials was sacrificed at each of three time points (Time 0, 2 hours, and 6 hours 20 minutes). To perform the cell counts, the clay was first allowed to settle in each vial. A sample of the supernatant was then diluted with BSM and plated on one quarter strength YEPG agar. The vial contents were resuspended, and a 1 mL sample of the suspension was diluted with a solution consisting of 8 mL BSM and 1 mL 10% sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$) to disperse the cells and clay. Further dilutions with BSM were made as needed followed by plating, again on one quarter strength YEPG agar.

The second test for change in cell numbers was a simple optical density (O.D.₅₄₆) reading of the vial supernatants. Readings were taken after a period of 3 -1/2 hours, during which samples were shaken continuously on an orbital shaker at 100 rpm. Four sets containing single samples of four clay quantities were used (0.0 g, 0.1 g, 0.4 g, and 1.0 g). Each vial received 4 mL of SSM, 0.5 mL SSM containing 25 mg L⁻¹ naphthalene, and 1 mL of YEPG containing approximately 3×10^8 cells of strain HK44 prepared by Growth Procedure II.

4.2.6.5 Test for External Mass Transfer Limitations

In this experiment, the mixing rate of the sample vial contents was varied to effect a change in the mass transfer rate of substrate to organisms as measured by change in light response. Three sets (identified as Racks 1, 2, and 3) were made up of single samples of four clay quantities (0.0 g, 0.1 g, 0.4 g, and 1.0 g). A 4 mL volume of SSM was

added to each sample vial. A 0.5 mL volume of SSM containing 25 mg L⁻¹ naphthalene was then added to the vials in racks 1 and 2. All three racks were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, all three racks of vials received 1 mL of YEPG containing approximately 3x10⁸ cells of strain HK44 prepared by Growth Procedure II. The vials in Rack 3 then received 0.5 mL SSM containing 25 mg L⁻¹ naphthalene, and that rack was left to stand with no mixing or shaking. Rack 1 was placed on the orbital shaker at 100 rpm, and the vials in Rack 2 were vortexed continuously. Bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.6 Test for Intraparticle Diffusion

This experiment was designed to test whether differences in light response could be attributed to intraparticle diffusion of naphthalene during the 24 hour equilibration period. It was hypothesized that intraparticle diffusion would cause a decrease in light response due to reduced quantity of immediately available naphthalene. Two sets (identified as Racks 1 and 2) were made up of single samples of four clay quantities (0.0 g, 0.1 g, 0.4 g, and 1.0 g). A 4 mL volume of SSM was added to each sample vial. A 0.5 mL volume of SSM containing 25 mg L⁻¹ naphthalene was then added to the vials in Rack 1. Both racks of vials were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, both racks of vials received 1 mL of YEPG containing approximately 3x10⁸ cells of strain HK44 prepared by

Growth Procedure II. The vials in Rack 2 then received 0.5 mL SSM containing 25 mg L⁻¹ naphthalene. Bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.7 Effect of Change in Clay:Microorganism Ratio

This experiment was designed to measure the effect on light response caused by starting with two different population densities. Two sets (identified as Racks 1 and 2) were made up of single samples of six clay quantities (0.0 g, 0.005 g, 0.05 g, 0.1 g, 0.2 g, and 0.4 g). A 5 mL volume of MBSM containing 25 mg L⁻¹ naphthalene was added to each sample vial. Vials in both racks were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, vials in Rack 1 received approximately 4×10^8 cells of strain HK44 prepared by Growth Procedure I. The vials in Rack 2 received approximately 10^{10} cells of strain HK44, also prepared by Growth Procedure I. Bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.8 Addition of Agents to Change Clay Aggregate Stability

Two experiments were conducted to observe the effect on light response of the addition of clay aggregate stabilizing or de-stabilizing agents. In the first experiment, three racks were set up containing single samples of two clay quantities (0.0 g and 1.0 g). Rack 1 vials received 4 mL BSM containing 25 mg L⁻¹ naphthalene. Rack 2 vials received 3.5 mL BSM containing 25 mg L⁻¹ naphthalene plus 0.5 mL 10% sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$). Rack 3 vials received

4 mL BSM containing 25 mg L-1 naphthalene plus 5.0 μ L of a 1:1 mixture of C₁₂E₄ and C₁₂E₂₃ polyoxyethylene (PE) nonionic surfactants. Vials in all three racks were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, all vials received 1 mL of YEPG containing approximately 3×10^8 cells of strain HK44 prepared by Growth Procedure II.

In the second experiment, three racks were set up containing single samples of four clay quantities (0.0 g, 0.1g, 0.4g, and 1.0 g). Vials in all three racks received 4 mL SSM containing 25 mg L-1 naphthalene. The vials were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, Rack 2 vials received 5.0 μ L of C₁₂E₄. All vials then received 1 mL of YEPG containing approximately 3×10^8 cells of strain HK44 prepared by Growth Procedure II. After 0.75 hours, Rack 3 vials received 5.0 μ L of C₁₂E₄. In both experiments, bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.9 Test for Particle Size Effects

This test was designed to determine whether sand particles would have the same effect on light response as clay particles. Three sets (identified as Racks 1, 2, and 3) were made up of single samples of four sand quantities (0.0 g, 0.1 g, 0.4 g, and 1.0 g). The sand used was white quartz sand, -50 +70 mesh (Sigma, St. Louis, MO). A 4 mL volume of SSM was added to each sample vial. A 0.5 mL volume of SSM containing 25 mg L⁻¹ naphthalene was then added to the vials in racks 1 and 2. All three racks were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, all three racks of vials received 1 mL of YEPG

containing approximately 3×10^8 cells of strain HK44 prepared by Growth Procedure II. The vials in Rack 3 then received 0.5 mL SSM containing 25 mg L^{-1} naphthalene, and that rack was left to stand with no mixing or shaking. Rack 1 was placed on the orbital shaker at 100 rpm, and the vials in Rack 2 were vortexed continuously. Bioluminescence was measured as described in Section 4.2.6.1 above.

4.2.6.10 Experiments Using Immobilized Cells

Two experiments were conducted using immobilized cells to observe the effect of eliminating contact between cell surfaces and clay particles. Immobilized cells were prepared as described in Section 4.2.4. In the first experiment, two sets were made up of single samples of four clay quantities (0.0 g, 0.1 g, 0.4 g, and 1.0 g). A 4 mL volume of SSM was added to each sample vial. A 0.5 mL volume of SSM containing 25 mg L^{-1} naphthalene was then added to the vials in Rack 1. Vials in both racks were mixed well and allowed to equilibrate for 24 hours. At the beginning of the assay, both racks of vials received 1.5 g of freshly made alginate beads containing a total of approximately 10^9 cells of strain HK44. The cell density of the original suspension and the weight of the beads resulting from 20 mL of cell suspension plus 40 mL of alginate were used to calculate the volume of original cell suspension in 1.5g beads, and thus the number of cells per vial. The vials in Rack 2 then received 0.5 mL SSM containing 25 mg L^{-1} naphthalene, and that rack was left to stand with no mixing or shaking. Rack 1 was placed on the orbital shaker at 100 rpm.

In the second experiment, one set of triplicate samples was made up of four clay quantities (0.0 g, 0.1 g, 0.4 g, and 1.0 g). A 4 mL volume of SSM was added to each sample vial followed by addition of 0.5 mL of SSM containing 25 mg L⁻¹ naphthalene. Vials were vortexed and allowed to equilibrate for 24 hours. At the beginning of the assay, all vials received 1.5 g of freshly made alginate beads containing a total of approximately 10⁹ cells of strain HK44. All vials were placed on the orbital shaker at 100 rpm. In both experiments, bioluminescence was measured as described in Section 4.2.6.1 above.

5.0 RESULTS - ABIOTIC STUDIES

5.1 Specific Surface Area and Particle Size Analyses

For each of the six potassium-saturated clays, the external specific surface area, as measured by low-temperature nitrogen adsorption, was lower than the value published in van Olphen and Fripiat (1979), although still comparable (Table 4). Some reduction in measurable surface area was expected due to agglomeration of the clay particles during the pretreatment process. In particular, freeze-drying caused the formation of large clumps which had to be broken up by hand manipulation with a stainless steel spatula.

The results of the Coulter Counter particle size analyses for pretreated and untreated SAz-1 are shown in Table 5. The approximate particle size range for the clay mineral fraction, given as 0.2 - 2 μm , was considered to be sufficient information for the other five clays. As was indicated by the surface area analyses, the pretreatment process caused an agglomeration of particles such that the measured sizes are significantly greater for the pretreated than for the untreated sample. The size distribution for the untreated sample also indicates a degree of agglomeration when compared with the size range of 0.2 - 2 μm expected for the clay mineral fraction. Accuracy of the particle size distribution may have been affected by use of a large-sized counting aperture relative to the size of the sample particles. The counting electrolyte may also have induced some particle flocculation.

Table 4. Nitrogen adsorption/BET surface area for source clays.
(all measurements in $\text{m}^2 \text{g}^{-1}$)

	Measured Value †	Published value ††
KGa-1	9.45 ± 0.01	10.05 ± 0.02
KGa-2	22.15 ± 0.09	23.50 ± 0.06
PFl-1	109.34 ± 0.34	136.35 ± 0.31
SAz-1	95.41 ± 1.05	97.42 ± 0.58
SHCa-1	55.67 ± 0.30	63.19 ± 0.50
STx-1	62.48 ± 0.17	83.79 ± 0.22

† Measured values are for K^+ saturated clays

††van Olphen, H. and J.J. Fripiat. 1979. *Data Handbook for Clay Materials and other Non-Metallic Minerals*. Pergamon Press, Oxford. pp. 13-34.

Clay identification key:

KGa-1	-Kaolinite, well crystallized
KGa-2	-Kaolinite, poorly crystallized
PFl-1	-Attapulgit, Florida
SAz-1	-Montmorillonite, Arizona (Cheto)
SHCa-1	-Hectorite, California
STx-1	-Montmorillonite, Texas

Table 5. Particle size distribution for SAz-1 montmorillonite.

Volume percent greater than measured size:	Particle Size Distribution (μm)	
	Pretreated	Untreated
10.0	25.56	7.03
25.0	18.94	6.29
50.0	12.55	5.38
75.0	8.17	4.30
90.0	5.68	3.46

5.2 Adsorption Isotherms

Data were fit to the Freundlich form of the adsorption isotherm, given by the equation

$$\frac{x}{m} = KC^{1/n}$$

where $\frac{x}{m}$ is the mass of sorbed compound per unit mass of adsorbing material, C is the equilibrium solution concentration of the compound in mass per unit volume, K is an empirical constant describing adsorption capacity, and n is a second empirical constant related to adsorption intensity. The values obtained for K and $1/n$ are presented in Table 6. Testing of the KGa-1 kaolinite was eliminated to reduce the size of the matrix. Due to an error, an ionic strength of 0.02 was inadvertently used in the aluminum PFI-1 adsorption isotherm instead of the ionic strength of 0.1 which was specified by the experimental matrix. Additionally, an ionic strength of 0.1 for the aluminum electrolyte solution produced a precipitate at pH 5.5 and above. Therefore, an ionic strength of 0.05 was used for the aluminum SHCa-1 adsorption isotherm, and aluminum was eliminated from the STx-1 test conditions. One adsorption isotherm procedure was conducted using blank vials containing no clay to determine the extent of losses due to volatilization or adsorption to the vial surfaces.

It can be seen from the low K values in Table 6 that naphthalene exhibited very little affinity for pure clay surfaces under all of the experimental matrix conditions. Raw data and isotherm plots are included in Appendix A.

Table 6. Adsorption isotherm data for naphthalene on five source clays.

Clay	Dominant Cation	pH	Ionic Strength	Adsorption Capacity, K (cm ³ g ⁻¹)	Percent of mass in solution after adsorption †
KGa-2	K ⁺	7	0.1	0.299	94.2
	Ca ⁺⁺	5.5	0.01	0.030	97.7
	Al ⁺⁺⁺	4	0.01	0.215	92.0
PFI-1	K ⁺	4	0.1	0.499	92.3
	Ca ⁺⁺	5.5	0.01	0.172	94.5
	Ca ⁺⁺	7	0.01	0.576	90.6
	Al ⁺⁺⁺	5.5	0.02	0.064	95.1
SAz-1	K ⁺	4	0.01	0.588	99.8
	K ⁺	5.5	0.1	0.000	104.5
	Ca ⁺⁺	4	0.1	0.001	97.2
	Al ⁺⁺⁺	7	0.01	0.000	104.9
SHCa-1	K ⁺	7	0.01	1.401	83.1
	Ca ⁺⁺	7	0.1	0.323	86.7
	Al ⁺⁺⁺	5.5	0.01	0.440	85.2
	Al ⁺⁺⁺	5.5	0.05	0.799	77.6
STx-1	K ⁺	5.5	0.01	1.128	87.0
	Ca ⁺⁺	4	0.01	0.211	90.1
	Ca ⁺⁺	4	0.1	0.079	94.9
Blank (no clay)	K ⁺	5.5	0.01	N/A	98.8

†Percent of radiolabeled naphthalene still in the solution phase after the 24 hours equilibration period

5.3 Discussion

Several observations can be made regarding the results of the abiotic experiments. Concerning the specific surface analysis, it should be noted that the nitrogen-BET method does not measure the interlayer surface area of expanding clays such as montmorillonite or hectorite (Bohn *et al*, 1985). However, these internal surfaces may not be available for sorption of naphthalene. Ogram *et al* (1985) stated that water intercalated by montmorillonite excluded 2,4-D which was in solution. Structurally, 2,4-D consists of a single benzene ring with a $-OCH_2COOH$ group in the one position, and one chlorine atom each in the two and four positions. It is reasonable to assume that naphthalene, consisting of two fused benzene rings, might also be excluded from interlayer surfaces.

While comparative K values for sorption of naphthalene on clays could not be found in the literature, experimentally determined values of $12 \text{ cm}^3 \text{ g}^{-1}$ (Mihelcic and Luthy, 1991) and $5.8 \text{ cm}^3 \text{ g}^{-1}$ (DiGrazia, 1991) have been reported for sorption on whole soils. As discussed in Section 2.3.1, the sorption of hydrophobic compounds has been correlated with the organic carbon content of whole soils. The low K values obtained here are therefore consistent with what could be expected for pure clay minerals containing negligible organic carbon.

An important consideration in measuring adsorption of a compound is the time at which the system is assumed to have achieved equilibrium conditions. Karickhoff and Morris (1985) reported that for PAH's, 35 to 60% of the equilibrium sorption onto natural sediments was achieved in minutes with apparent equilibrium being reached after a

few hours. Whereas rapid equilibration has been similarly reported in other studies (Karickhoff *et al*, 1979; Rogers *et al*, 1980), other researchers have observed a slow sorption phenomenon which may last from days to months (Ball, 1989; Robinson *et al*, 1990). For this study, the time frame of interest was the range of 24 to 36 hours required to set up and perform a biological experiment. The data presented here should be considered in light of that time frame, and care should be taken in applying these results to a dissimilar set of conditions.

The parameters that went into construction of the experimental matrix for the adsorption isotherm experiments can all, in theory, have an effect on sorption to clay mineral surfaces. For a nonpolar, nonionic, hydrophobic compound such as naphthalene, differences in sorptive capacity would most likely result from changes in the degree of hydration of the clay surfaces. It has been reported that in soil-water systems, polar and/or electrostatically charged mineral surfaces preferentially adsorb water, becoming strongly hydrated and prohibiting interaction with nonionic organic contaminants (Guerin and Boyd, 1992). Stotzky (1986) suggested that highly hydrophobic molecules may be repelled from the surface of a clay mineral with a high CEC, as a result of the hydration of the exchangeable cations. In an aqueous system, the cations in solution are surrounded by a hydrated sphere, the size of which varies with the radius of the ion. The three cations used in this study, placed in order of decreasing hydrated radius are $\text{Al}^{+++} > \text{Ca}^{++} > \text{K}^{+}$, therefore, the aluminum-saturated clay should be more hydrated than calcium- or potassium- saturated.

Conditions that would cause hydrated cations to come into closer contact with the clay surface can also be thought of as increasing the repulsion of hydrophobic molecules. As ionic strength increases, cations are brought closer to clay surfaces as electrostatic attractive forces overcome diffusive forces. Changes in pH alter surface charges through gain or loss of hydrogen ions from Al-OH functional groups. It has been observed that in acidic solutions, anions are retained by kaolinite indicating the presence of positive sites (Bohn *et al*, 1985). Likewise, with decreasing pH, cation retention and surface hydration would decrease. Therefore, sorption capacity for a hydrophobic compound would be expected to be higher at lower pH.

From the standpoint of the hydration of the clay surface, some consistency in the data can be seen by looking at the measured sorption capacities of the three expanding clays as presented in Table 7. The parameter that had the most consistent effect on adsorption capacity was the ionic strength of the solution. For both the SHCa-1 hectorite and the STx-1 montmorillonite, the highest adsorption capacities were measured at the lowest ionic strength. This appears to be an effect that may be limited to pure mineral surfaces. Stauffer and MacIntyre (1986) observed that a variation of the ionic strength over three orders of magnitude had little effect on naphthalene sorption on a whole soil. In the case of the SAz-1 montmorillonite, it may be that the large CEC overshadowed the effect of the ionic strength. Decreasing pH did not appear to correlate with increased adsorption capacity as expected, nor did the hydrated radius of the dominant cation. While the data presented here are insufficient to fully determine the mechanism of

Table 7. Adsorption capacities and related parameters for the expanding clays.

Adsorption Capacity, K (cm ³ g ⁻¹)	Clay	CEC† (cmol kg ⁻¹)	Dominant Cation	pH	Ionic Strength
1.401	SHCa-1	43.9	K ⁺	7	0.01
1.128	STx-1	84.4	K ⁺	5.5	0.01
0.799	SHCa-1	43.9	Al ⁺⁺⁺	5.5	0.05
0.588	SAz-1	120.0	K ⁺	4	0.01
0.440	SHCa-1	43.9	Al ⁺⁺⁺	5.5	0.01
0.323	SHCa-1	43.9	Ca ⁺⁺	7	0.1
0.211	STx-1	84.4	Ca ⁺⁺	4	0.01
0.001	SAz-1	120.0	Ca ⁺⁺	4	0.1

†van Olphen, H. and J.J. Fripiat. 1979. *Data Handbook for Clay Materials and other Non-Metallic Minerals*. Pergamon Press, Oxford. pp. 13-34.

adsorption, they do provide an adequate characterization of the clay-naphthalene interaction for use in design of the biological experiments. Based on the results of the abiotic experiments, KGa-2 kaolinite and SAz-1 montmorillonite were chosen for use in the biodegradation studies. The negligible sorption capacity of SAz-1 for naphthalene at pH 7 allowed adsorption to be eliminated as a biodegradation rate-limiting mechanism under any of the experimental conditions used for the mineralization or bioluminescence tests. Although the KGa-2 kaolinite exhibited a slightly greater sorption capacity for naphthalene, it was selected for use in mineralization tests primarily for its low specific surface area.

6.0 RESULTS - MINERALIZATION

The quantities of clay used in the mineralization experiments were chosen to provide a comparison between similar clay volumes (1 g kaolinite compared with 1 g montmorillonite), and between similar external surface areas (4 g kaolinite compared with 1 g montmorillonite). Results are presented in Figure 2. Average mineralization rates at 6 hours were 0.14 day^{-1} for 1g kaolinite, 0.05 day^{-1} for 4g kaolinite, and 0.13 day^{-1} for 1g montmorillonite. For comparison, a review by DiGrazia (1991) of naphthalene mineralization rate constants in batch soil slurries gives a range of 0.002 to 3.8 day^{-1} . Naphthalene mineralization kinetics appeared similar for both the 1 g clay conditions, but were retarded in the presence of 4 g kaolinite. Both the rate and extent of mineralization were quite close to those reported by Korde (1991) for a solid to liquid mass ratio of 1:5 (i.e. between 60% and 80% mineralized within 12 hours). Although 1 g of montmorillonite possessed approximately the same external surface area as 4 g of kaolinite, the 4 g kaolinite system showed a slower mineralization rate, and less total mineralization over the 12 hours of the experiment. Therefore, it appeared that naphthalene mineralization was influenced by the total volume of solids rather than by clay-specific properties such as surface area or CEC.

As the ratio of 4 g kaolinite to 5 mL BSM (close to 50% clay by weight) produced a very thick slurry, oxygen diffusion must be

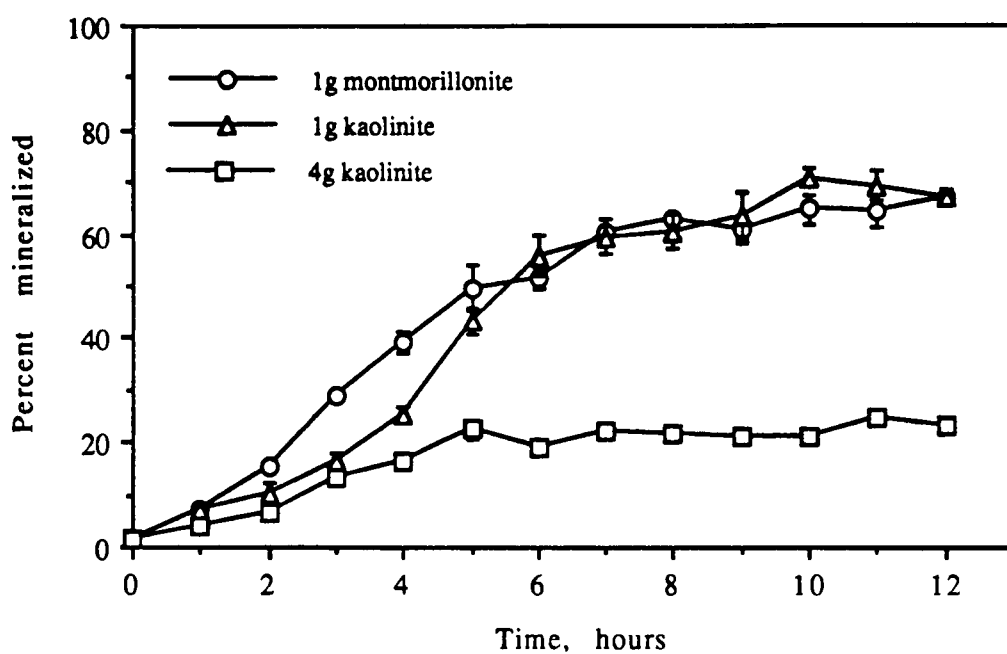


Figure 2. Naphthalene mineralization by strain 5R as measured by $^{14}\text{CO}_2$ production. Each vial contained 5 mL of BSM with 25 mg l^{-1} naphthalene and approximately 3×10^8 cells.

considered as a potentially limiting factor. Stotzky (1966a) suggested that oxygen diffusion was impaired by the viscosity of a clay suspension containing only 8% montmorillonite. As mentioned previously, the 4 g quantity of kaolinite was selected solely to provide surface area equivalent to 1 g montmorillonite. In subsequent experiments, a maximum montmorillonite to liquid ratio of 1:5 was used. Based on the rate and extent of mineralization that were observed for this proportion, oxygen diffusion was not expected to be a limiting factor at the 1:5 ratio.

The percent of naphthalene mineralized was obtained by dividing the radioactivity in dpm's recovered in the CO₂ traps by the total dpm's added to each experimental vial. A mass balance on radiolabeled naphthalene is therefore desirable to gauge the accuracy of the calculated percentage. A summary of the mass balances for all three clay conditions is presented in Table 8. Based on these mass balances, the combined efficiencies of the CO₂ trap, hexane, and water extractions were 94% for the KGa-2 + strain 5R samples, but only 72% for the SAz-1 + strain 5R samples. The lower mass balance for the SAz-1 montmorillonite prompted the additional step of combusting the samples. A significant portion of the radioactivity in the 5R-containing SAz-1 vials was recovered during oxidation, and that portion was observed to increase with time from 3,100 dpm per vial at Time 0, to 15,800 dpm per vial after 12 hours. As the adsorption isotherm data for SAz-1 demonstrated negligible sorption capacity for naphthalene, the mass balance may indicate the presence of a polar metabolite that has a binding affinity for montmorillonite, but not for kaolinite. The absence

Table 8. Mass balance (average dpm per vial) for mineralization assay.

	Degraders (Strain 5R)			Non-degraders (Strain PB2440)		
	1 g KGa-2	4 g KGa-2	1 g SAz-1	1 g KGa-2	4 g KGa-2	1 g SAz-1
DPM's recovered (average per vial)						
CO ₂ Trap	21,120	9,280	62,670	1,270	840	2,800
Extracted with hexane	21,620	42,680	34,160	48,440	57,320	110,480
Polar phase	3,200	N/A	5,690	1,750	N/A	4,640
Oxidized	N/A	N/A	12,350	N/A	N/A	2,650
Total dpm's recovered	45,940	51,960	114,870	51,460	58,160	120,570
Added initially	48,920	55,430	142,080	48,920	55,430	142,080
Percent recovered	93.9	93.7	80.8	105.2	104.9	84.9

of the oxidizable fraction in the non-degrading PB2440 vials would tend to confirm this suggestion, whereas the absence of a comparable fraction in the KGa-2 polar phase would indicate that unbound metabolite could be completely mineralized. For both the 5R and the PB2440 vials, the inability to recover a greater percentage by oxidation may have been due to volatilization losses during the air-drying step prior to combustion of the samples.

7.0 RESULTS - BIOLUMINESCENCE

7.1 Light Response Characterization

In the initial characterization experiments, distinct differences in the pattern of light output were observed between strain 5RL in salicylate-supplemented YEPG (Figure 3), and strain HK44 in naphthalene-supplemented MBSM (Figure 4A). In full-strength, unbuffered YEPG medium, strain 5RL exhibited a steady increase in light over a period of approximately 3-1/2 hours, after which there was a slight decrease followed by a sharp peak in light response up to 120 nAmps, and an equally sharp drop-off. The vial containing no clay showed the greatest light output until reaching about 50 nAmps, however, peak light levels for all six clay concentrations were within the range of 100 - 120 nAmps. The sharp drop-off appeared to correspond to rapidly decreasing pH levels within the vials, although the drop in pH was not positively identified as the cause of decreased light output. Hydrogen ion activities were observed to be higher with increasing clay content, which is consistent with previous suggestions that montmorillonite clay has significant buffering capacity (Stotzky, 1966b).

In one-quarter strength, unbuffered YEPG, strain 5RL again showed a gradual increase in light response, followed by a rapid increase in light output similar to the response with full-strength YEPG. Interestingly, the peak light response reached above 900 nAmps compared with 120 nAmps in the full-strength YEPG vials. Additionally,

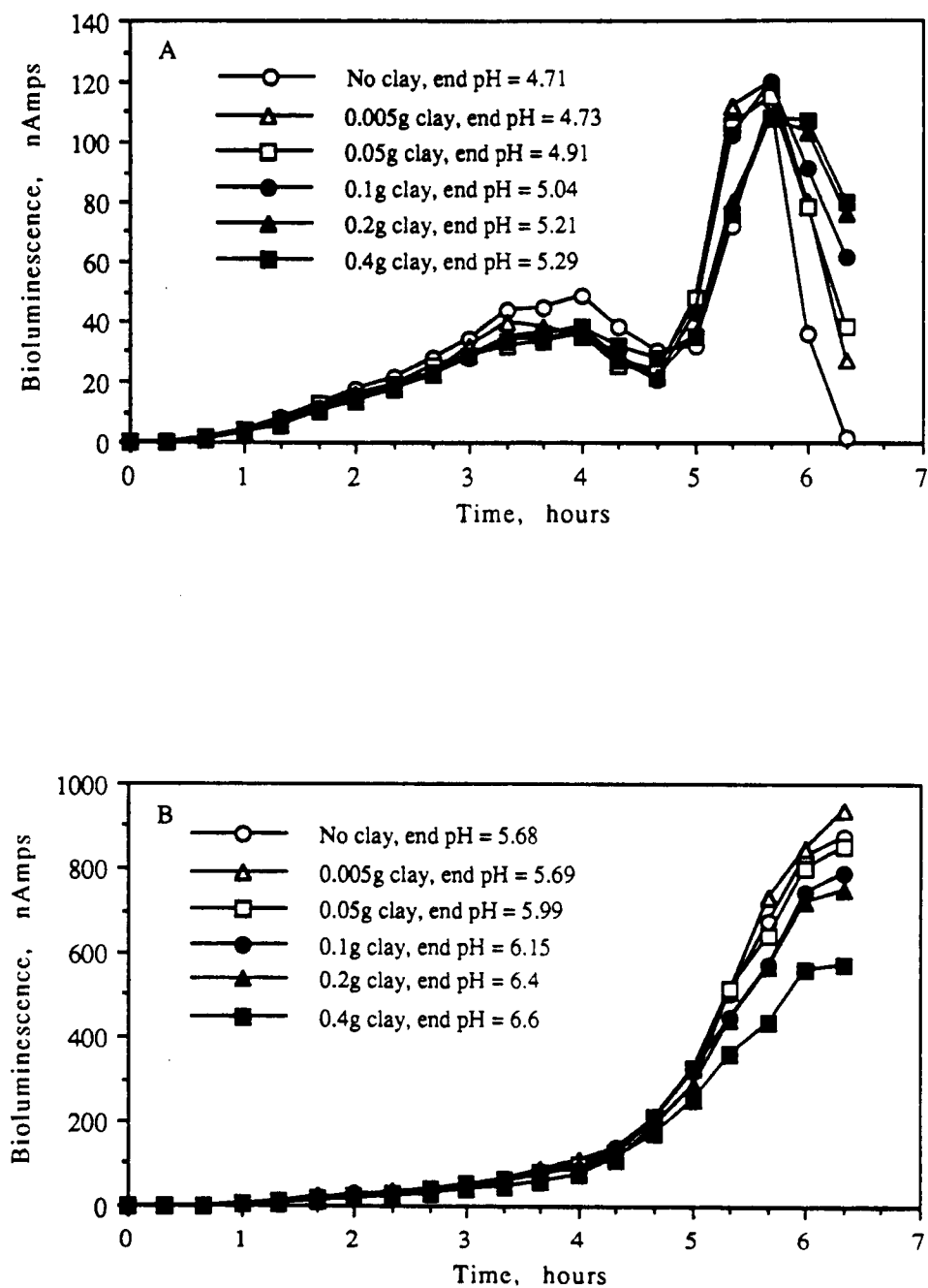


Figure 3. Bioluminescence response to salicylate (0.5 g L^{-1}) by strain 5RL: (A) in full-strength YEPG medium, and (B) in quarter-strength YEPG medium. 5 mL medium and 3×10^9 cells per vial.

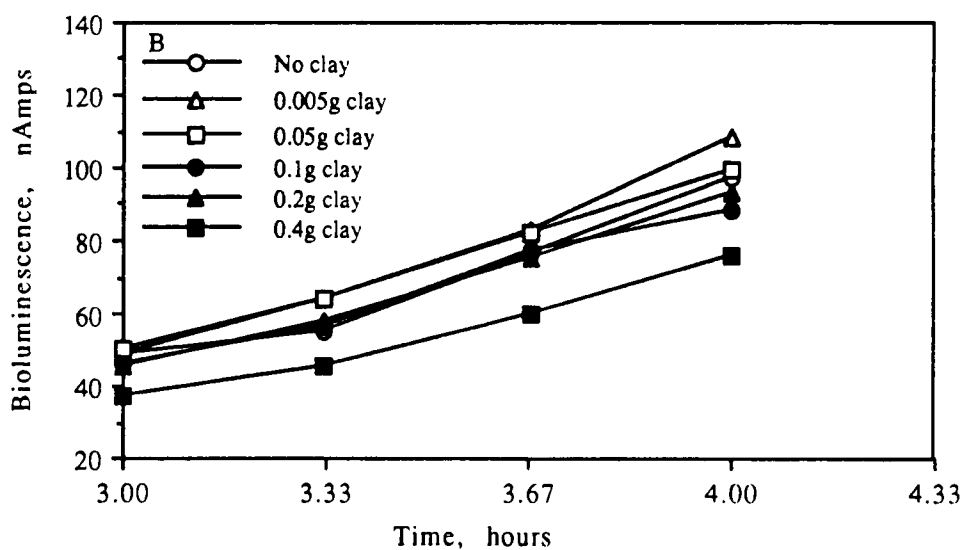
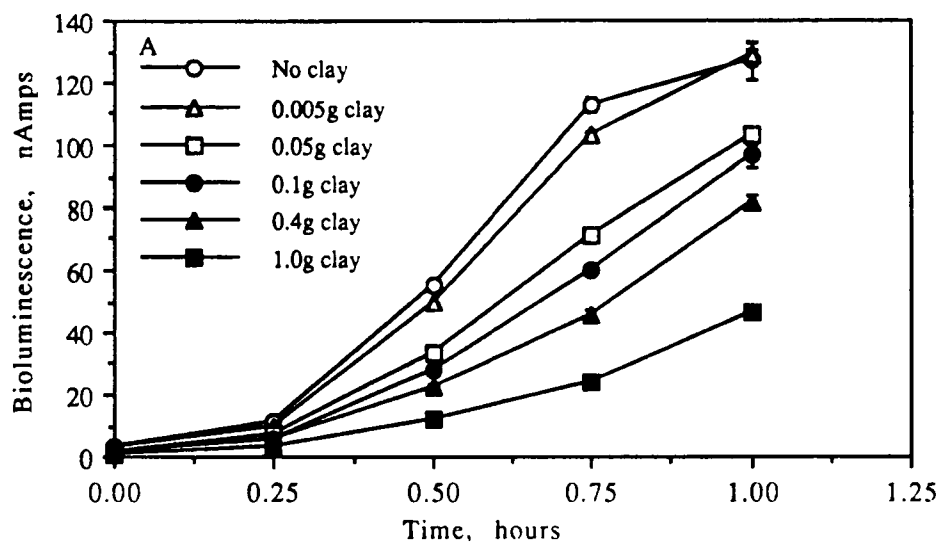


Figure 4. (A) Bioluminescence response to naphthalene by strain HK44. Each vial contained 3 mL MBSM with 3 mg L⁻¹ naphthalene, and 2mL YEPG with approximately 6x10⁸ cells. (B) Data taken from Figure 3B, hours 3 to 4.

the quarter-strength YEPG samples exhibited a greater range in the peak response values. Light output in the vials with 0.0 g to 0.05 g clay ranged from 850 - 950 nAmps, while in the 0.1 g and 0.2 g vials light output peaked at 750 - 800 nAmps, and in the 0.4 g vial, the highest light reading was 570 nAmps. In contrast with the full-strength YEPG vials, the differences in light output at the end of the 6-1/2 hour experiment did not appear to correspond to differences in pH.

The 5RL light response curves shown in Figure 3 shared two important characteristics. First, even at light levels below 25 nAmps, but particularly at high light levels, the relatively narrow range of light readings at different clay concentrations suggested that the presence of clay within the vials did not interfere with the light detection method. Visually, even while shaking, most of the clay particles appeared to remain near the bottom of the vial, below the level of the light probe. Second, the pattern of the light response was quite similar to that observed by Burlage and coworkers (1990) for *Pseudomonas putida* strain RB1351 in the presence of naphthalene. Burlage proposed that the particular light response pattern reflected a preferred slow growth rate for the utilization of naphthalene. This suggested the possibility that there may be a point at which light output is a function of cell processes not directly related to the concentration of naphthalene available to the organisms. With this possibility in mind, and for purposes of excluding any other changes in light that might be related to a transition between growth phases, it was decided to focus on the immediate effect of the clay particles by limiting subsequent experiments to a maximum two hour window.

In naphthalene-supplemented MBSM, as shown in Figure 4A, light response by strain HK44 was observed to decrease with increasing clay concentration. For comparative purposes, the rates of light output between 0.5 and 0.75 hours, in nAmps hr⁻¹, in order of increasing clay concentration, are 229.5, 213.6, 152.1, 127.9, 91.8, and 49.8. In comparison to the observations for strain 5RL in YEPG (Figure 3), a much wider range in light readings at different clay concentrations was observed. Figure 4B shows a one hour period out of the 5RL response data from Figure 3B in which the same approximate magnitude of light is produced as shown by HK44 in Figure 4A, but over a much narrower range. It appeared that the clay particles were interfering with the uptake of naphthalene as measured by light output. Data from the abiotic characterization showed that the interference was not likely to be caused by adsorption of naphthalene onto the clay. The effect, if real, must therefore be the result of some other clay-substrate or clay-microbe interaction.

Figure 5 presents the results of the bioluminescence response to four different concentrations of naphthalene in the absence of clay. Rates of light output between 0.5 and 0.75 hours, in nAmps hr⁻¹, in order of decreasing naphthalene concentration, are 434.1, 250.9, 29.2, and 40.8, with a rate of 2.5 nAmps hr⁻¹ in the absence of naphthalene. Here the effect of naphthalene concentration on the magnitude of the light response is clearly illustrated. Comparing Figure 5 with Figure 4A, it appears as if the effect on the light response of increasing clay concentration is similar to decreasing naphthalene concentration in

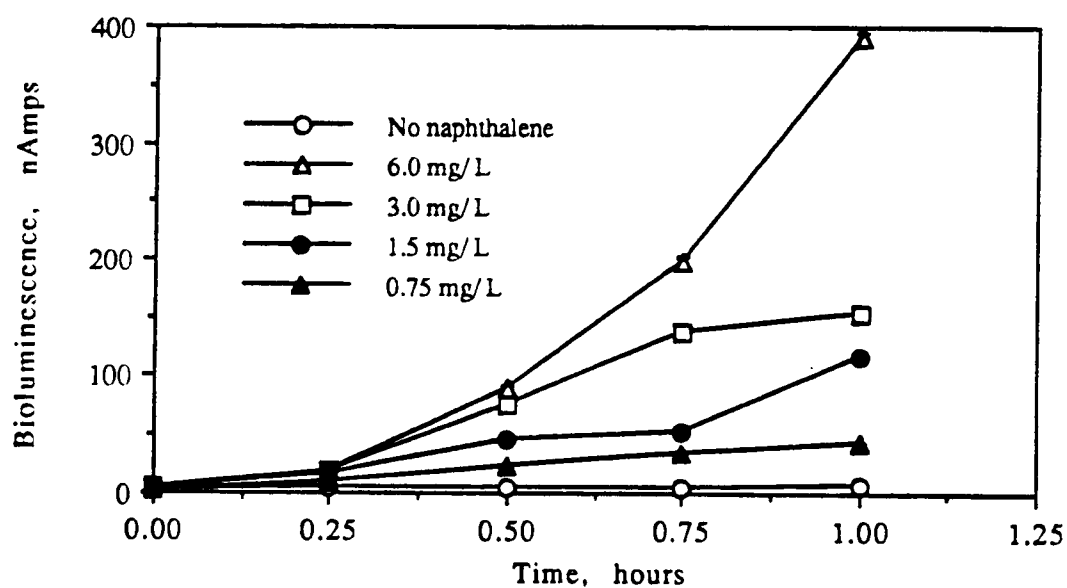


Figure 5. Bioluminescence response to naphthalene by strain HK44 in the absence of clay. Each vial contained 3 mL MBSM, and 2mL YEPG with approximately 6×10^8 cells. Concentrations of naphthalene in each vial are as indicated in the legend.

the absence of clay. Whether or not this is coincidental is discussed in Section 8.0.

7.2 Correlation of Bioluminescence with Mineralization

To verify that the decrease in light output with increasing clay concentration did in fact indicate a decrease in naphthalene uptake, the experiment was performed to correlate bioluminescence with mineralization. The light response that was measured in this experiment, presented in Figure 6, duplicated the characteristic pattern for strain HK44 described in Section 7.1 and shown in Figure 4A. Although the experimental conditions indicate that 0.2 g and 2.0 g clay were used, the 1:5 clay to liquid ratio was maintained, making these results comparable to those for 0.1 g and 1.0 g used in other experiments. The data for the concurrent mineralization assay show a pattern similar to that seen for the light response, with an increase in clay concentration corresponding to a decrease in the cumulative percent mineralized. To produce a comparable representation of cumulative light production, a numerical integration was performed to obtain the areas under the light response curves. The results for the mineralization assay are shown in Figure 7A, and the accompanying cumulative light output is presented in Figure 7B.

It appears from these results that the decrease in measured light with increasing clay concentration was not merely an artifact of the experimental method, but corresponded to a similar decrease in mineralization rate. However, although a qualitative correlation can be

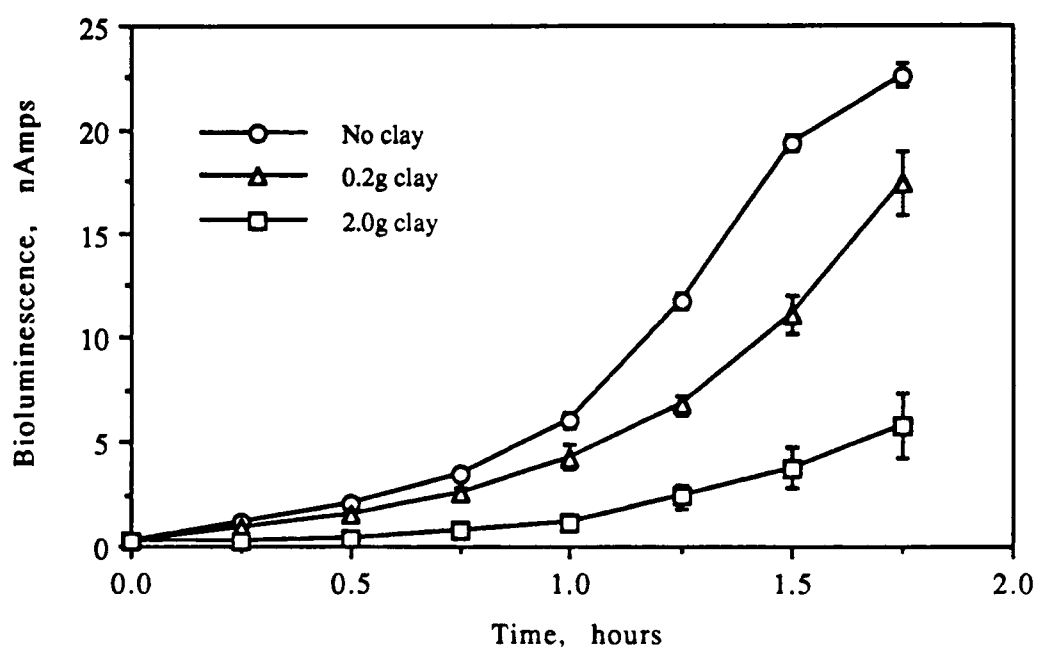


Figure 6. Bioluminescence response to naphthalene by strain HK-44 for correlation with mineralization. Each vial contained 9 mL MBSM with 3 mg L⁻¹ naphthalene, and 1 mL YEPG with approximately 6×10^8 cells.

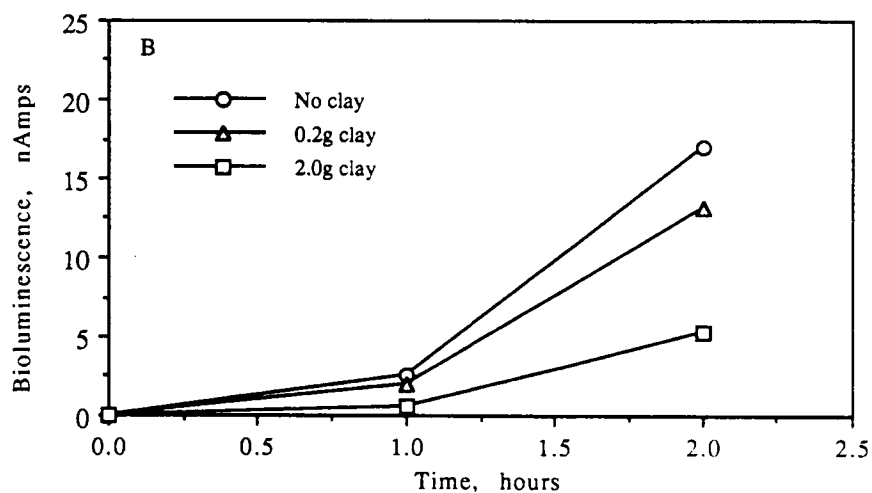
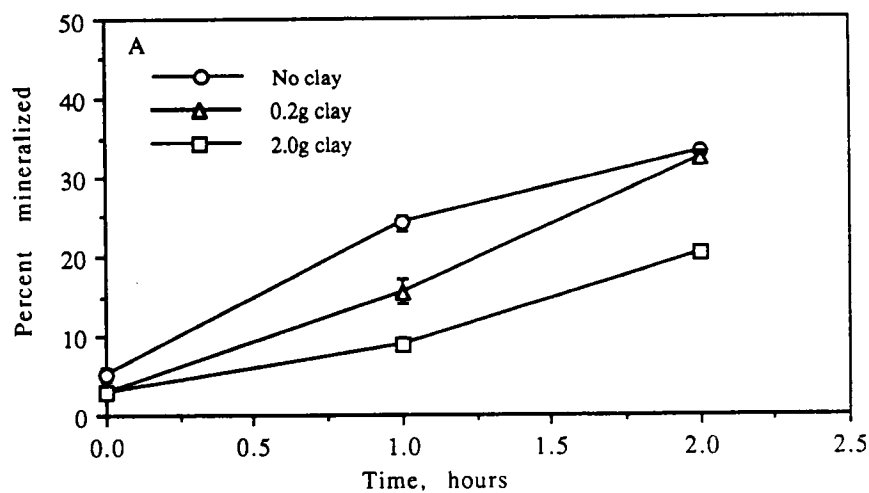


Figure 7. (A) Naphthalene mineralization by strain HK44 as measured by $^{14}\text{CO}_2$ production. Each vial contained 9 mL MBSM with 3 mg L^{-1} naphthalene, and 1 mL YEPG with approximately 6×10^8 cells. (B) Integration of data presented in Figure 6.

made, differences in the slopes of the lines suggest that the rate of light output does not directly duplicate the mineralization rate.

A comparison of mineralization and bioluminescence rates is provided in Table 9. These values show that in the first hour, both assays indicate decreased activity with increasing clay content. In the second hour however, the mineralization rate for the no clay condition is decreasing, the 0.2g condition is staying nearly constant, and the 2.0g condition is increasing. In contrast, the rates of light output for all three conditions are rapidly increasing. From these observations, it appears that after the first hour, light output becomes independent of the mineralization rate.

Table 9. Mineralization versus bioluminescence: comparative rates.

	Hour 0 to 1		Hour 1 to 2		Percent change†	
	Mineralization rate (mg hr ⁻¹)	Bioluminescence rate (nAmps hr ⁻¹)	Mineralization rate (mg hr ⁻¹)	Bioluminescence rate (nAmps hr ⁻¹)	Mineralization	Bioluminescence
No clay	0.0065	2.5	0.0024	14.5	-63.1	+480
0.2g	0.0042	2.0	0.0046	11.2	+ 9.5	+460
2.0g	0.0024	0.6	0.0031	4.7	+29.2	+683

$$\dagger \text{ Percent change} = \frac{(\text{Hour 1 to 2}) - (\text{Hour 0 to 1})}{(\text{Hour 0 to 1})} * 100$$

7.3 Test for Change in Cell Numbers

To determine whether the rapid increase in light output observed in previous experiments could be related to growth, experiments were performed to examine changes in cell numbers over the course of a typical bioluminescence assay. As this test was designed specifically to identify possible growth-related effects, the two-hour restriction on experiment length was not imposed. The light response that was measured in this experiment again demonstrated the characteristic pattern observed in previous experiments (Figure 8).

Changes in viable cell numbers, as measured by plate counts, are shown for three different clay concentrations relative to the results for the no clay condition (Figure 9, A-C). None of the three clay concentrations showed any consistent increase or decrease in cell numbers relative to the no clay condition. In addition, there was no significant difference between the cell numbers associated with the clay suspension versus cell numbers in the supernatant. This would suggest that the cells were suspended rather than attached to the clay, and that the cells remained suspended after the clay settled out. If a significant number of cells had become attached to the clay, the cell numbers counted for the suspension samples should have been consistently higher for the suspension than for the supernatant. Although the data for the 0.1 g and 0.4 g samples do in fact show higher cell numbers for the suspension than for the supernatant, there is sufficient variation in the Time 0 values to cast some doubt as to the accuracy of the plate count method. Jannasch and Pritchard (1972) reported that separation of bacterial cells from particles by sub-lethal

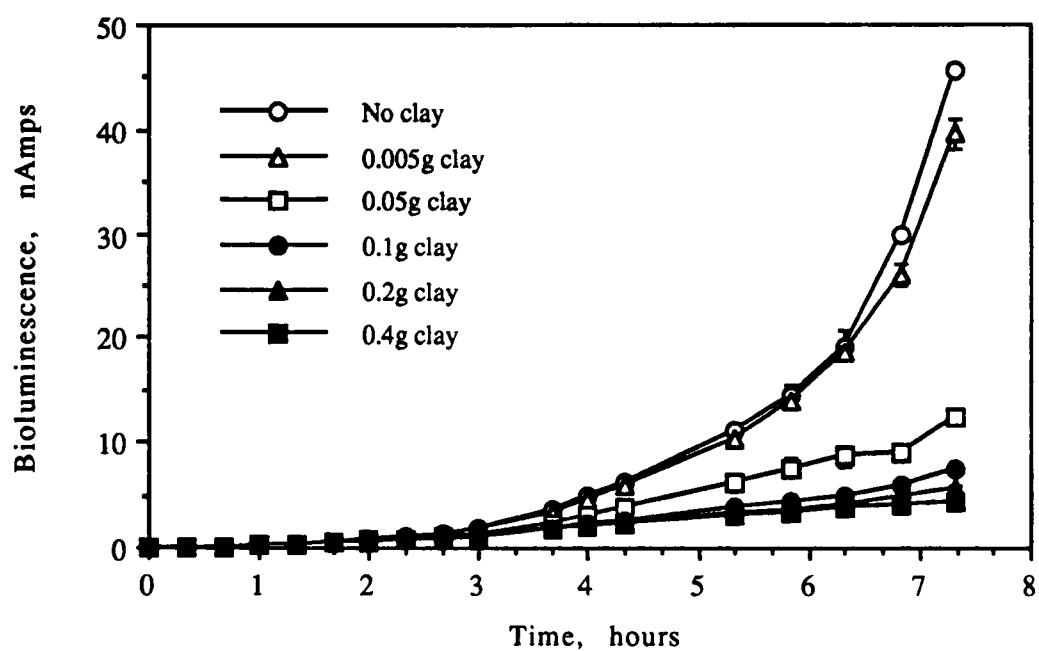


Figure 8. Bioluminescence response to naphthalene by strain HK44 in test for change in cell numbers. Each vial contained 5 mL MBSM with 25 mg L^{-1} naphthalene and approximately 10^9 cells.

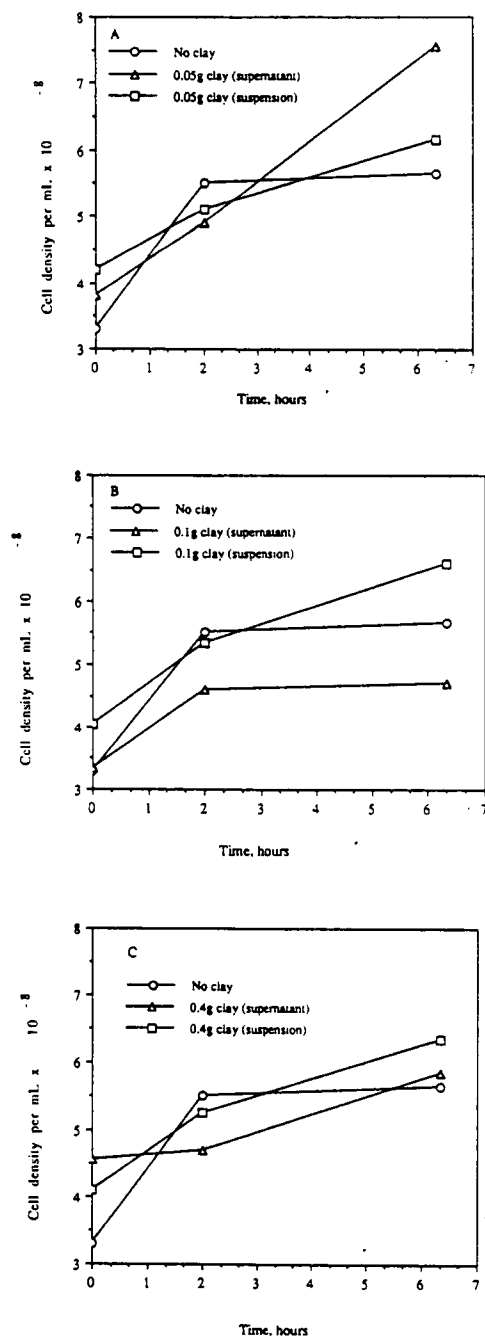


Figure 9. Change in cell numbers as determined by plate counts, corresponding to the bioluminescence data for three of the clay concentrations presented in Figure 8: (A) 0.05g clay, (B) 0.1g clay, and (C) 0.4g clay.

concentrations of surface active agents was not effective in producing the plate counts that were expected based on microscopic observations. If plate counts are accepted, results indicate at most one doubling in cell numbers over a period of 6 hours, even for the no clay condition. For comparison, OD₅₄₆ readings for a culture of HK44 in liquid YEPG during log phase suggest a doubling time of approximately 1.5 hours. It would appear that over the course of this experiment, naphthalene utilization, as determined by light output, was primarily for cell maintenance rather than for growth.

It can also be seen that although the greatest increase in cell numbers generally occurred over the first two hours, this corresponded to a long lag period in the light response, whereas the greatest increase in light output, most noticeably in the no clay vial, was not accompanied by a corresponding increase in viable cell numbers.

The possibility was considered that the lack of growth in the plate count experiment was due to cell culture and preparation by Growth Procedure I. This appeared to be confirmed by the optical density readings in a separate experiment that used cells prepared by Growth Procedure II. A typical bioluminescence assay was prepared by placing in each vial 1mL of YEPG containing approximately 3×10^8 cells, or 6×10^7 cells mL⁻¹ after dilution with the medium in the vial. The optical density of the supernatant after 3.5 hours was measured to be between 0.2 and 0.3 in all replicates for four different clay concentrations (Figure 10A). The final OD readings represented a cell density of between 2×10^8 and 3×10^8 cells mL⁻¹; a 4- to 5-fold increase, or approximately two doublings. This increase in cell numbers is

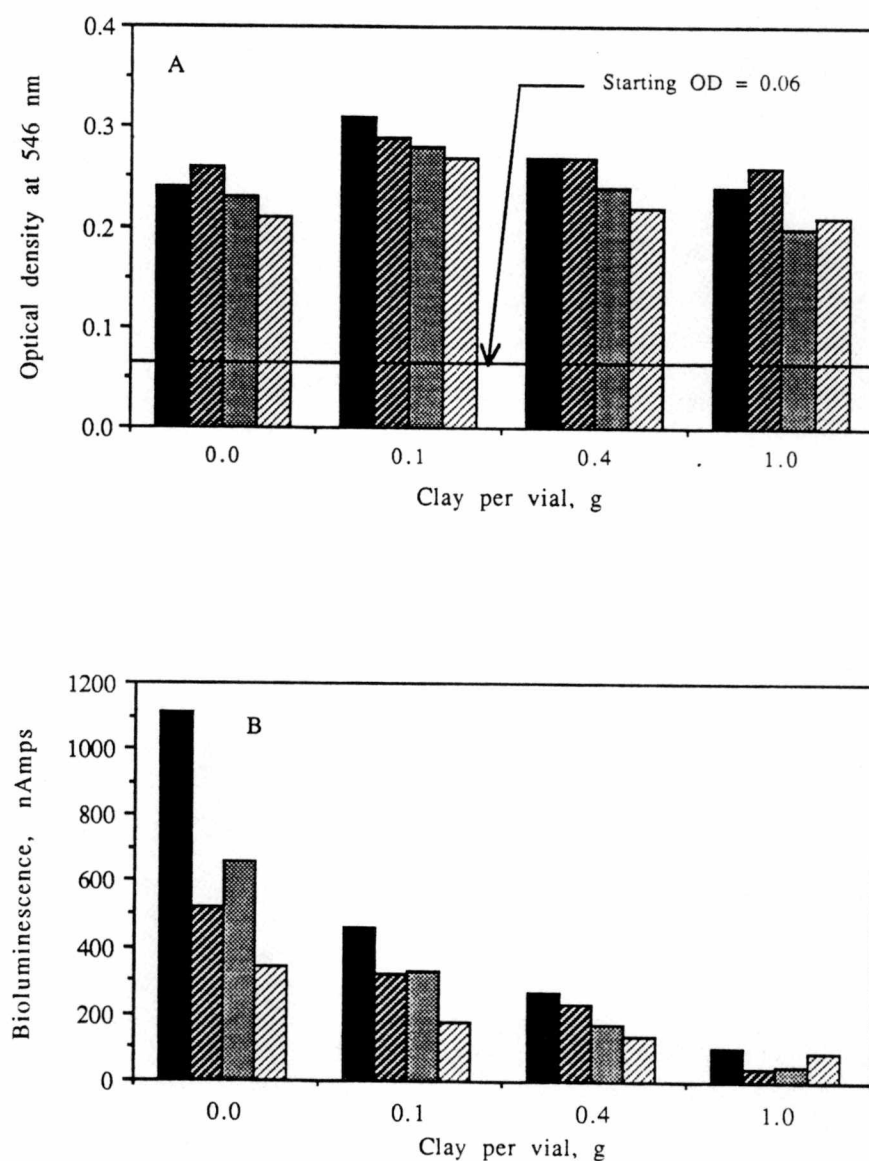


Figure 10. (A) Optical density readings of vial supernatants after a typical bioluminescence assay of 3.5 hours duration (4 replicates for each clay concentration). (B) Bioluminescence responses for the same vials 15 minutes prior to OD reading. Each vial started with 5 mL SSM with 2.5 mg L⁻¹ naphthalene and an OD₅₄₆ of 0.06 corresponding to approximately 6 × 10⁷ cells mL⁻¹ strain HK44.

consistent with log phase predictions. An interesting observation is that although all four clay concentrations had similar OD values, the bioluminescence readings taken 15 minutes earlier are very different (Figure 10B). Compared with the performance by the cells prepared by Growth Procedure I, the Growth procedure II cells produced ten times more light in half the time with ten times less naphthalene in solution. The use of samples prepared by both procedures demonstrated that the characteristic variation in light output with clay content, as well as the rapid increase in light after a period of exposure, occurred both with and without growth. It was also apparent that cells prepared by Growth Procedure II provided a more homogeneous and better characterized population. The differences in light output among different clay concentrations did not correspond to differences in growth, and the rapid increase in light output could not be attributed to a sudden change in cell numbers.

7.4 Test for External Mass Transfer Limitations

Considering the procedure that had been established for mixing of vial contents (placement of vial racks on an orbital shaker), it seemed possible that the observed differences in light output could be a result of the mixing regime created within the vials. As discussed in Section 2.3.2, the addition of particles to a suspension may cause an increase in viscosity with an accompanying decrease in the mass transfer coefficient of a dissolved species. However, the use of three dramatically different mixing speeds was not observed to change the pattern of light output. All three conditions exhibited the characteristic

decrease in light output with increasing clay concentration (Figure 11).

The rates of light output between 0.75 and 1.0 hours, in nAmps hr⁻¹ were:

	<u>No mixing</u>	<u>Shaken</u>	<u>Vortexed</u>
No clay	14.8	34.4	35.6
0.1g	10.0	18.4	13.2
0.4g	10.8	14.8	6.8
1.0g	6.8	13.6	0.6

Lower levels and rates of light output were observed in the vials that were not mixed, however, the relative differences between the different clay concentrations were no more pronounced than for the other two mixing conditions. One important observation was that for the 1 g sample that was vortexed, enough particles became suspended in the region of the vial where the light probe was placed to interfere with bioluminescence detection. This suggests that possibility that an actual increase in light output in the shaken or vortexed vials was masked by increased clay suspension. However, such an effect should have been clearly displayed in the no clay vials. Therefore, it can be assumed that with the exception of the 1 g vortexed sample, total particle suspension did not significantly interfere with light detection.

It was expected that if external mass transfer had been a significant factor, an increase in mixing speed would have increased the mass transfer rate of naphthalene to the cells which would have been evidenced by an increase in light production. The lack of such an observation suggests that the external mass transfer of naphthalene

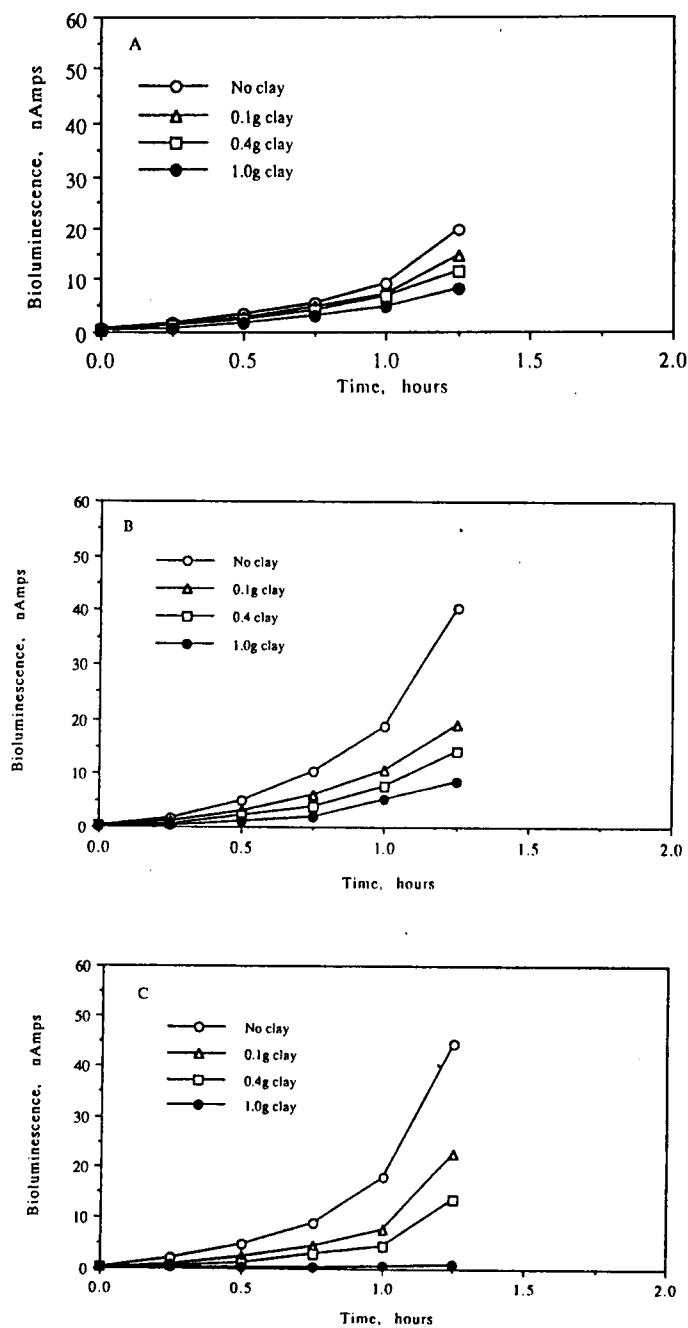


Figure 11. Bioluminescence response to naphthalene by strain HK44 in test for mass transfer limitations: (A) no mixing of vial contents, (B) vials placed on an orbital shaker at 100 rpm, and (C), vials continuously vortexed. Each vial contained 5 mL volume of SSM with 2.5 mg L^{-1} naphthalene and 1 mL of YEPG with approximately 3×10^8 cells.

across the film layer between the bulk solution and the organism is not a limiting factor for biodegradation. It is also implied that the addition of clay particles in suspension up to 1 g in 5 mL does not affect viscosity to the point of significantly increasing the film layer thickness. As bioluminescence is oxygen dependent, the results would also indicate that there is no limiting film resistance for oxygen transfer.

7.5 Test for Intraparticle Diffusion

It was expected that the effect of intraparticle diffusion would be to retard the rate of mass transfer of naphthalene to the cells (and decrease light production) by slowing the bulk diffusion rate. As the quantity of intraparticle micropores would be expected to increase with increasing clay concentration, so the bulk diffusion rate and light output would be expected to decrease with an increase in clay particles. An experiment was designed to determine whether a 24 hour contact time was sufficient to allow significant diffusion of naphthalene into intraparticle micropores. If light output was the same for all clay concentrations in a set of vials to which fresh naphthalene had been added immediately before the assay, there would be strong evidence for intraparticle diffusion in the 24 hour vials. The similar light levels in both racks of vials suggest that the same quantity of naphthalene was immediately available for microbial uptake (Figure 12).

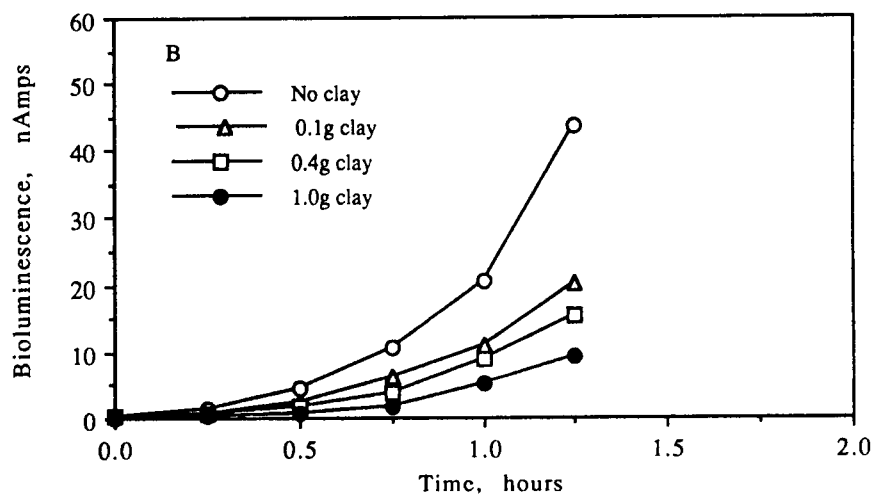
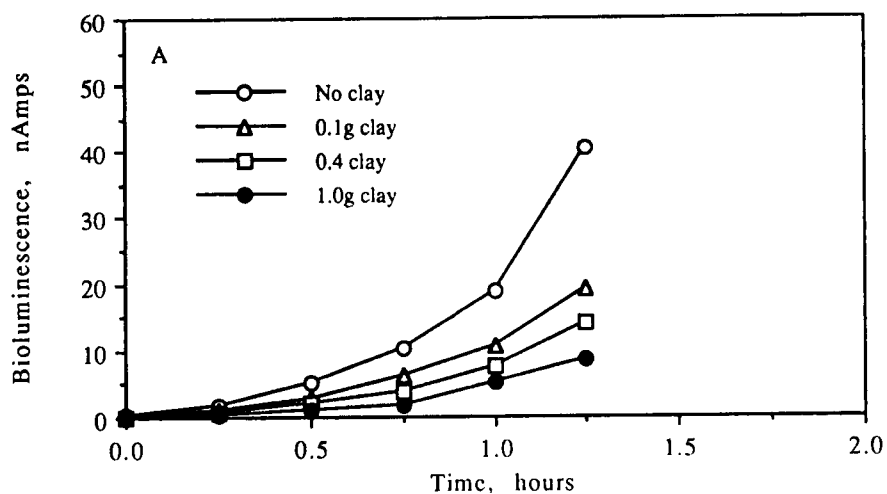


Figure 12. Bioluminescence response to naphthalene by strain HK44 in test for intraparticle diffusion: (A) naphthalene applied to clay slurry 24 hours before assay, and (B), naphthalene applied immediately before assay. Each vial contained 5 mL volume of SSM with 2.5 mg l^{-1} naphthalene and 1 mL of YEPG with approximately 3×10^8 cells.

The rates of light output between 1.0 and 1.25 hours, in nAmps hr⁻¹ were:

	<u>24 hour application</u>	<u>Immediate application</u>
No clay	86.4	91.2
0.1g	34.0	36.8
0.4g	26.8	26.0
1.0g	13.2	17.2

Based on the response of the no clay condition, the lower rate of light output in the vials exposed to naphthalene for 24 hours can most likely be attributed to volatile loss of naphthalene from solution over the exposure period. These results appear to rule out intraparticle diffusion as a rate-limiting mechanism in the time frame of this experiment.

7.6 Effect of Change in Clay:Microorganism Ratio

This experiment was designed to determine whether the inhibitory effect of clay on light output could be altered by changing the clay:microorganism ratio. It was thought that if the clay were exerting a direct effect on the cells, a change in population density might override that influence. The immediately observable effects of increased cell density were an acceleration in the characteristic light response pattern, and a higher level of total light output. The complete data set for the 5 hour experiment is presented in Figure 13 to show the level of light output achieved in the high cell density vials relative to that in the vials with fewer cells. An obvious feature demonstrated by

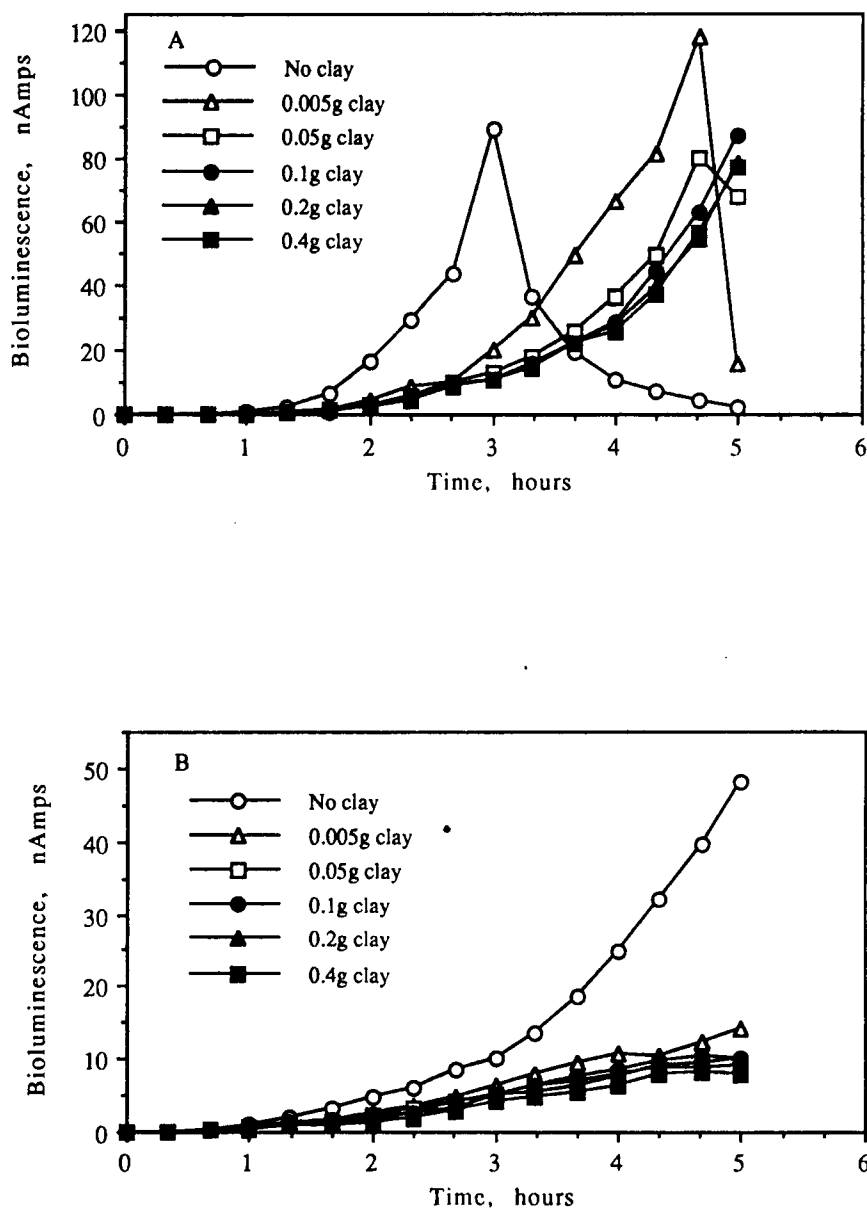


Figure 13. Bioluminescence response to naphthalene by strain HK-44 in test for effect of change in clay: microorganism ratio: (A) approximately 10^{10} cells added per vial, and (B), approximately 4×10^8 cells added per vial. Each vial contained 5 mL MBSM with 25 mg L^{-1} naphthalene.

the high-density cells is the rapid increase and drop-off seen in the initial characterization experiments. Although the high cell density vials achieved greater total light output, when the data are normalized to light output per million organisms as shown in Figure 14, it can be seen that the higher cell density light response actually translated to less light per organism. Therefore, while the activity of the population as a whole was accelerated in the presence of large numbers of cells, the activity of the individual organisms appeared to be reduced, possibly due to competition on an individual basis for nutrients and oxygen.

If the time windows are chosen to show a two hour period and a range of light output up to 50 nAmps, responses by the two cell densities look remarkably similar with respect to clay concentration effect (Figure 15). This is not surprising in light of the volume of clay particles compared to the volume of organisms. Assuming cylindrical organisms $0.5\ \mu\text{m}$ in diameter and $1.0\ \mu\text{m}$ in length, total cell volume per vial would be $19.6 \times 10^6\ \mu\text{m}^3$ for 10^8 cells, and $19.6 \times 10^8\ \mu\text{m}^3$ for 10^{10} cells. In comparison, assuming an average particle density of $2.65\ \text{g cm}^{-3}$, the specific volume of the clay would be $3.8 \times 10^{11}\ \mu\text{m}^3\ \text{g}^{-1}$. Only the volume of particles in $0.005\ \text{g}$ clay is small enough to come close to the volume of 10^{10} cells. For all the larger clay quantities, the volume of particles is orders of magnitude greater than the volume of cells. It is therefore not surprising that increasing the cell density from 10^8 to 10^{10} cells did not affect the characteristic light response pattern. As it becomes impractical to grow even larger numbers of cells in a single batch culture, it was decided that changing the clay:microbe ratio was

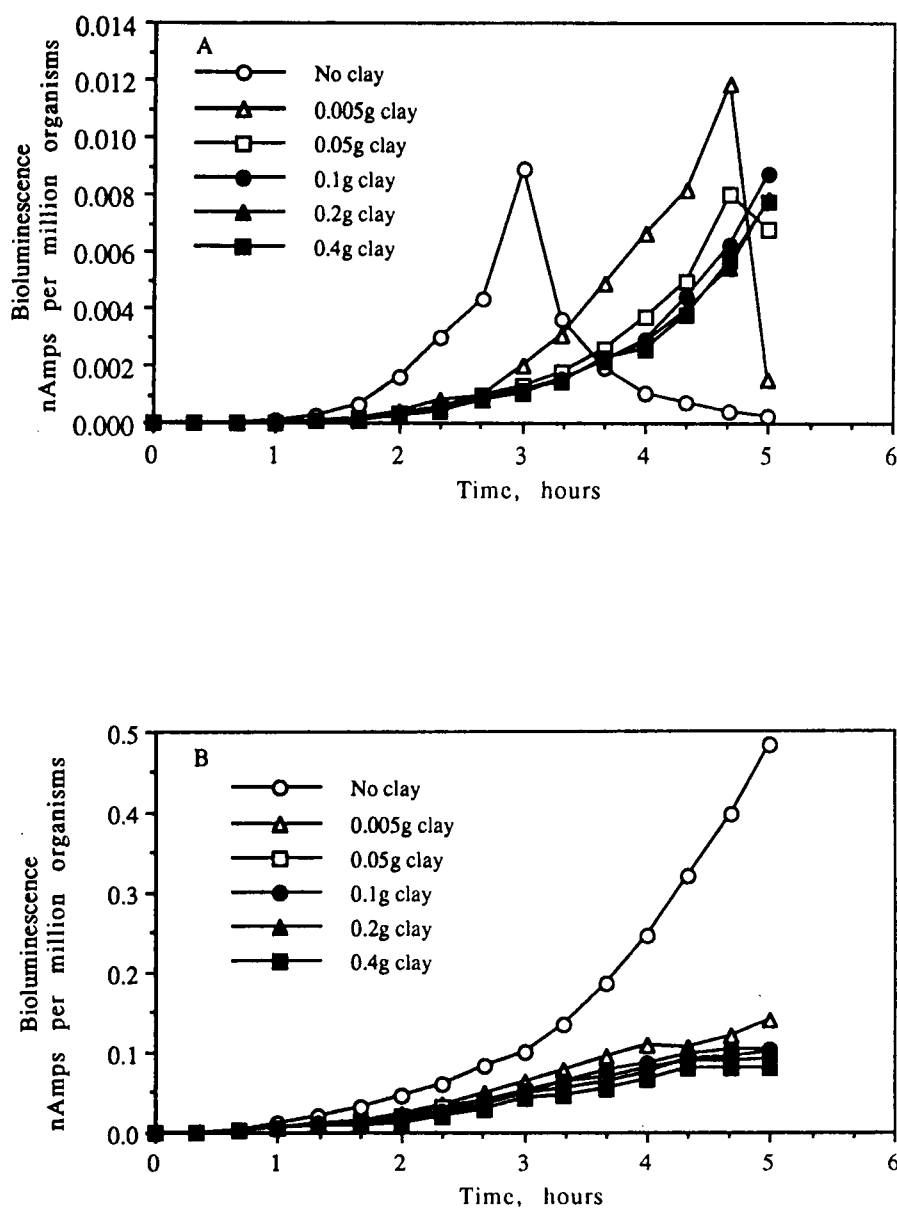


Figure 14. Bioluminescence response to naphthalene by strain HK44, normalized to light per million organisms: (A) approximately 10^{10} cells added per vial, and (B), approximately 4×10^8 cells added per vial. Each vial contained 5 mL MBSM with 25 mg L^{-1} naphthalene.

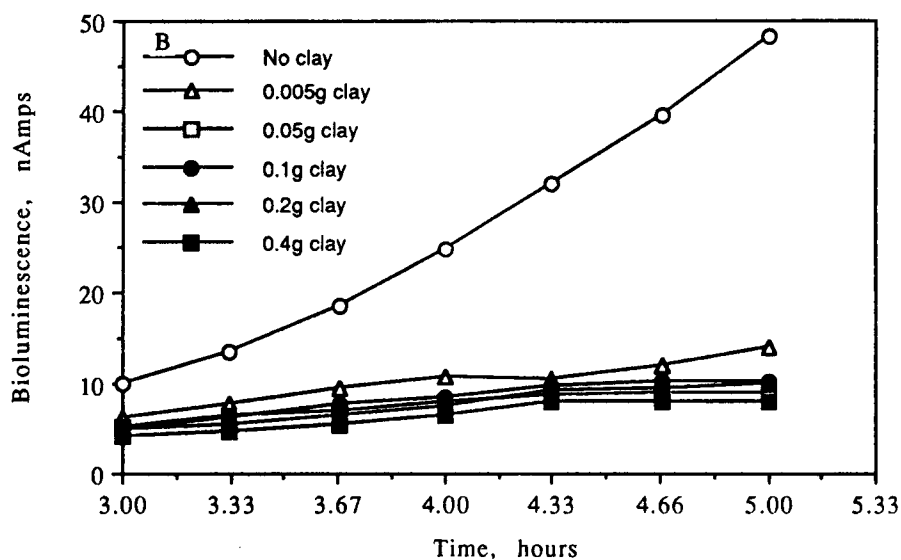
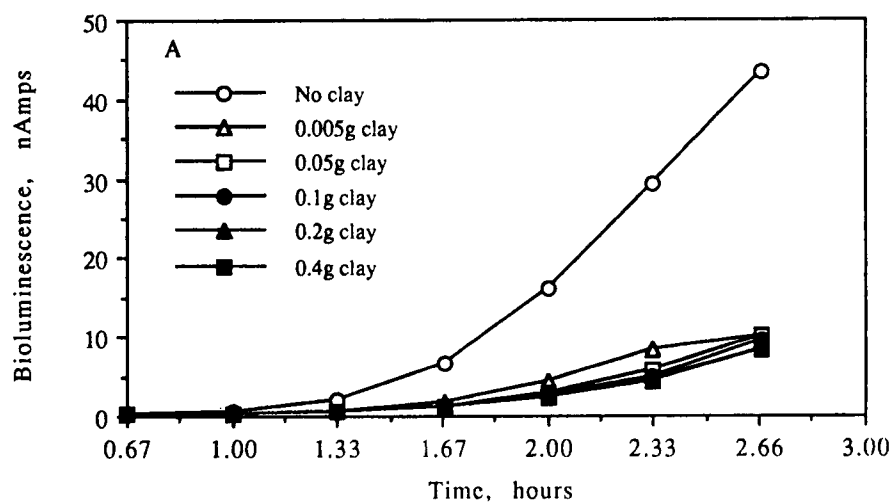


Figure 15. Bioluminescence response to naphthalene by strain HK-44, 2 hour time windows taken from Figure 12: (A) approximately 10^{10} cells added per vial, and (B), approximately 4×10^8 cells added per vial. Each vial contained 5 mL MBSM with 25 mg L⁻¹ naphthalene.

not a satisfactory method of examining direct effects of clay particles on the organisms.

7.7 Addition of Agents to Change Clay Aggregate Stability

A different experimental approach started with the premise that a clay-microbe association did exist, and that it was reversible. In an attempt to disrupt any reversible clay-microbe association, this experiment examined the effect of adding either sodium pyrophosphate (an aggregate destabilizer) or a 1:1 mixture of C₁₂E₄ and C₁₂E₂₃ polyoxyethylene (PE) nonionic surfactants (an aggregate stabilizer). As can be seen in Figure 16, neither additive caused a significant change in the light output for samples containing 1 g clay. The only observable differences were in the no clay vials where the sodium pyrophosphate caused an initial increase in light output over the no-additive vial, and the surfactant acted to decrease the light response.

The cause for the enhancement of light output by sodium pyrophosphate is not known. Three possible mechanisms, based on observations by Laha and Luthy (1991), can be suggested for the effect of the surfactant: (1) the surfactant may have been preferred over naphthalene as a substrate, (2) the surfactant concentration may have been toxic to the organisms, and (3) naphthalene may have been hidden from microbial attack within aggregates of surfactant molecules known as micelles. It is unlikely that within the time frame of the experiment, the organisms would have produced the proper enzymes for metabolism of the surfactant, and less likely that the surfactant would have been preferred over naphthalene for which the organisms possessed a

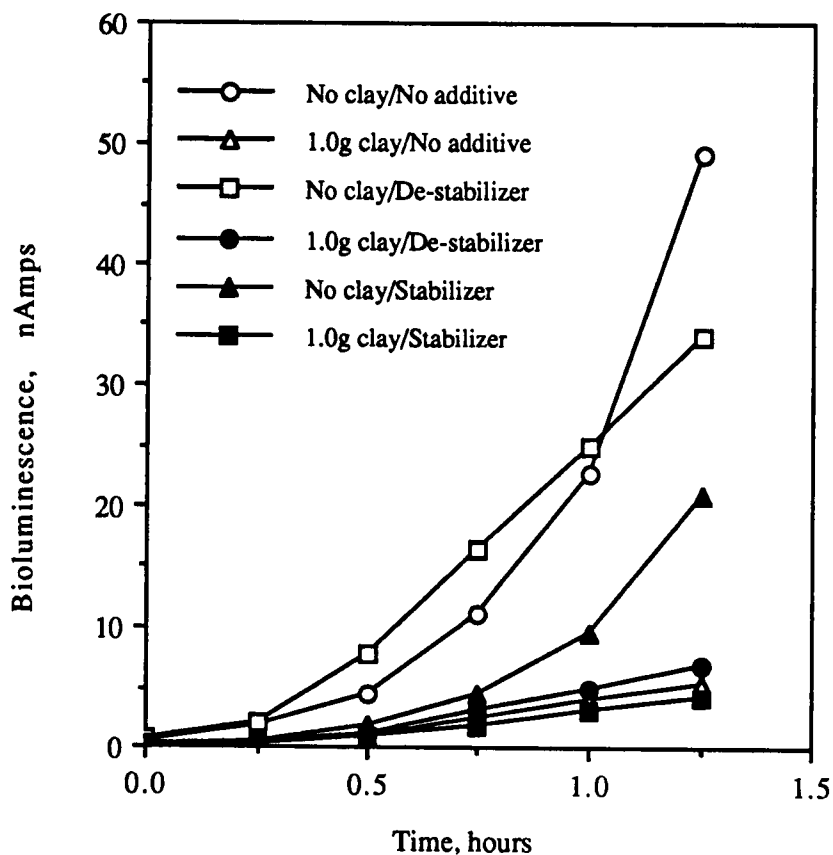


Figure 16. Bioluminescence response to naphthalene by strain HK44 in first test with agents to change aggregate stability. Stabilizer - 0.05% C₁₂E₄:0.05% C₁₂E₂₃ polyoxyethylene nonionic surfactants. Destabilizer - 1.0% sodium pyrophosphate (Na₄P₂O₇). Each vial contained 4 mL BSM with 25 mg L-1 naphthalene and 3x10⁸ cells.

readily inducible pathway. It also seems unlikely that the level of light that was detected would have been observed had the surfactant been toxic to the organisms. The third possibility appears the most likely. The clay particles that usually appeared as a milky suspension in the untreated vials, were visually aggregated into small flocs in the presence of the surfactant. Laha and Luthy (1991) observed that the presence of soil changed the surfactant micellization pattern due to surfactant sorption onto the soil, resulting in an aqueous-phase surfactant concentration that was considerably less than the total added. Therefore, it would seem likely that the clay acted to sorb the surfactant and reduce the aqueous phase to a level below the critical micelle concentration (CMC), i.e., approx. $8.3 \times 10^{-4}\%$ (v/v) for $C_{12}E_4$ (Laha and Luthy, 1991). Without the formation of surfactant micelles, the aqueous phase naphthalene concentration, and the resulting light output, remained relatively unaffected. In the absence of clay however, micelles were formed which enclosed a portion of the naphthalene rendering it unavailable to the organisms, and causing a reduction in bioluminescence.

A slightly different result was observed in the experiment in which 0.1% $C_{12}E_4$ was added at two different time points (Figure 17). The level of the light response in the clay-containing vials seemed to be unaffected by the addition of the surfactant, while the vial without clay showed decreased light output.

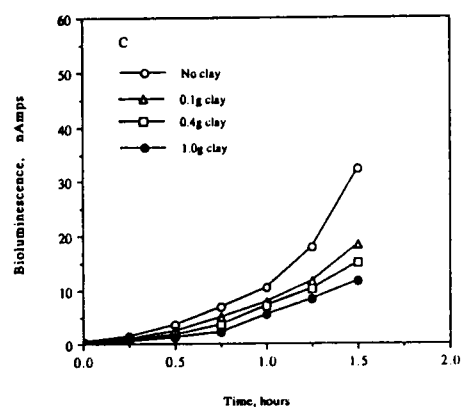
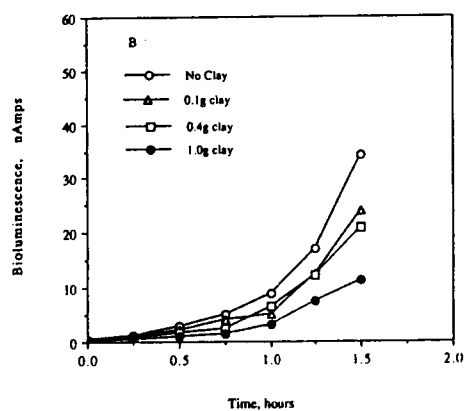
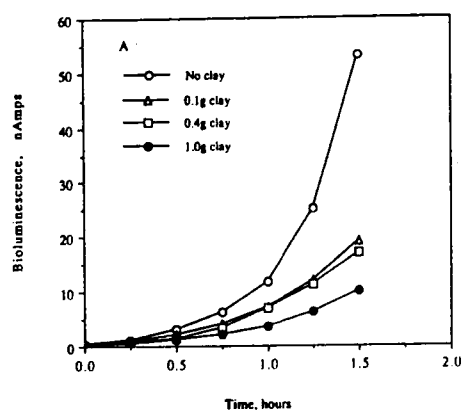


Figure 17. Bioluminescence response to naphthalene by strain HK44 in second test with agents to change aggregate stability: (A) no stabilizer, (B) stabilizer added at 0 hours, and (C) stabilizer added at 0.75 hours. Stabilizer - 0.05% C₁₂E₄:0.05% C₁₂E₂₃ polyoxyethylene nonionic surfactants. Each vial contained 4 mL SSM with 25 mg L⁻¹ naphthalene and 3x10⁸ cells.

The rates of light output between 0.75 and 1.0 hours, in nAmps hr⁻¹ were:

	<u>No stabilizer</u>	<u>Stabilizer added at 0 hours</u>	<u>Stabilizer added at 0.75 hours</u>
No clay	22.3	15.3	13.4
0.1g	12.8	3.8	11.3
0.4g	13.8	16.0	14.1
1.0g	6.0	6.4	13.6

An increased rate of light output was observed for the 1g clay condition receiving the stabilizer at 0.75 hours, however, no such enhancement was observed for any of the vials receiving the stabilizer at Time 0. Neither of the aggregate stability experiments demonstrated any consistent effect on the level of light produced in the clay-containing vials, indicating that the cause of reduced light output in the presence of clay was still operating. It was still possible that a reversible clay-microbe interaction was present that was unaffected by the stabilizing or de-stabilizing agents. Another possibility was the existence of a more permanent, adhesive clay-microbe contact.

7.8 Test for particle size effects

A unique characteristic of the clay mineral fraction is the presence of sub-micron sized, colloidal particles. In previous experiments, it was observed that only the smallest particles remained continuously suspended during the assay. To determine whether only

the suspended particles were responsible for the reduction in bioluminescence, an experiment was performed using sand particles (-50 +70 mesh, or 200 to 300 μm size range) instead of clay. The sand did not have the effect on light output observed for the different clay concentrations (Figure 18). The rates of light output between 1.25 and 1.5 hours, in nAmps hr^{-1} were:

	<u>No mixing</u>	<u>Shaken</u>	<u>Vortexed</u>
No sand	101.2	81.2	72.4
0.1g	132.0	80.8	72.8
0.4g	83.2	68.4	61.6
1.0g	90.8	68.0	45.6

At three different mixing speeds, light output in the sand-containing vials was similar to the level observed in the no sand samples. Reduced light rates and levels were observed in the vortexed vials, not only with respect to quantity of sand, but also relative to the vial sets that were shaking or at rest. It is possible that the vortex speed damaged the cells, especially in the presence of the sand particles, although this was not observed in the vortexed vials in the earlier mass transfer experiment. Nonetheless, the results of this test suggest that the inhibited light production seen in the presence of clay may be caused in some way by the smallest, continuously-suspended particles.

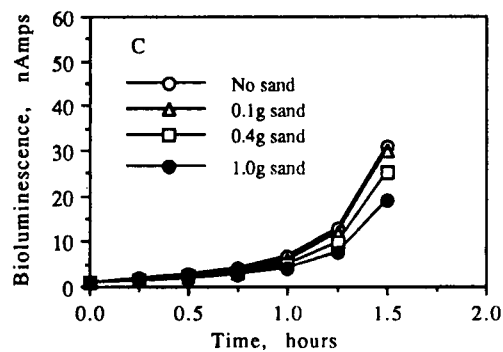
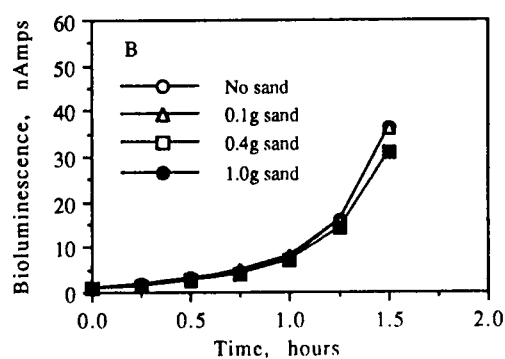
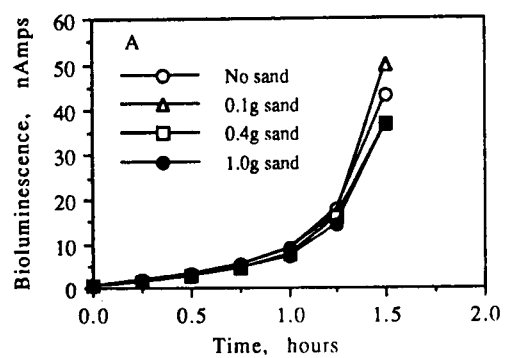


Figure 18: Bioluminescence response to naphthalene by strain HK44 in test for particle size effects: (A) no mixing of vial contents, (B) vials place on an orbital shaker at 100 rpm, and (C), vials continuously vortexed. Each vial contained 4 mL SSM with 2.5 mg L^{-1} naphthalene and 3×10^8 cells.

7.9 Experiments Using Immobilized Cells

At this point, a direct clay-microbe interaction still remains to be demonstrated, although adsorption, external mass transfer, intraparticle diffusion, and growth-related changes have been eliminated as possible mechanisms for the observed reduction in light output in the presence of an increasing quantity of clay particles. Other experiments have suggested that if a clay-microbe interaction exists, it may be adhesive, and involve sub-micron sized particles. It was therefore decided to perform a set of experiments using cells immobilized in alginate, which would prevent any direct contact between the microbes and the clay.

When immobilized cells were used, a very different pattern in the light response was observed, as can be seen in Figures 19 and 20. The rates of light output in Figure 20, between 1.0 and 1.25 hours, in nAmps hr^{-1} , in order of increasing clay concentration, are 52.0, 57.6, 62.3, and 85.2. The vials without clay no longer exhibited the highest level of light, in fact, they were observed to have the least light output. The range in light response was narrower than had been observed with suspended cells. In addition, there was a definite difference in light output depending on whether the vials were shaken or at rest, indicating a possible external mass transfer limitation at the boundary of the alginate beads. It is almost certain that in changing from suspended cells to immobilized cells, the effect of eliminating direct cell-clay contact was confounded by the effect of adding another mass transfer parameter, that of diffusion of naphthalene into the bead. The

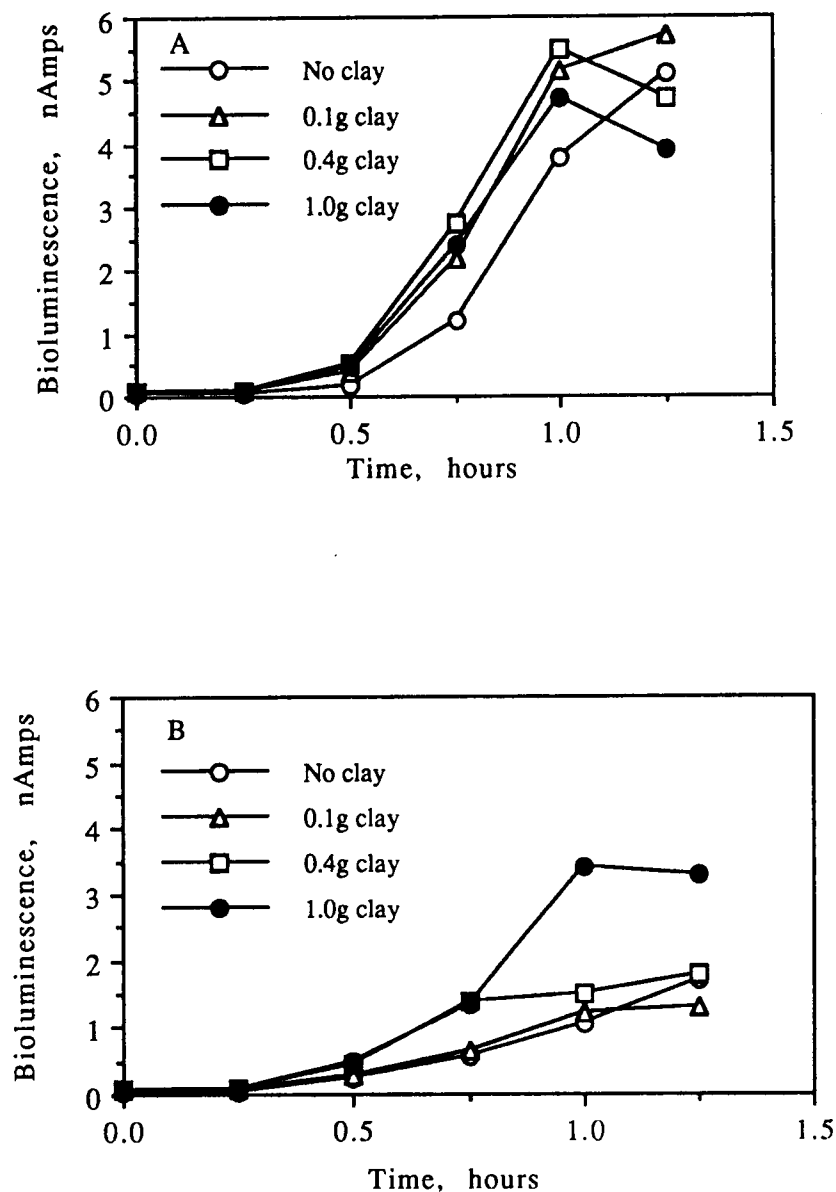


Figure 19. Bioluminescence response to naphthalene by strain HK-44 immobilized in alginate beads (first test): (A) vials placed on an orbital shaker at 100 rpm, and (B) no mixing of vial contents. Each vial contained 4 mL SSM with 2.5 mg L^{-1} naphthalene and 1.5 g of freshly made alginate beads with a total of approximately 10^9 cells.

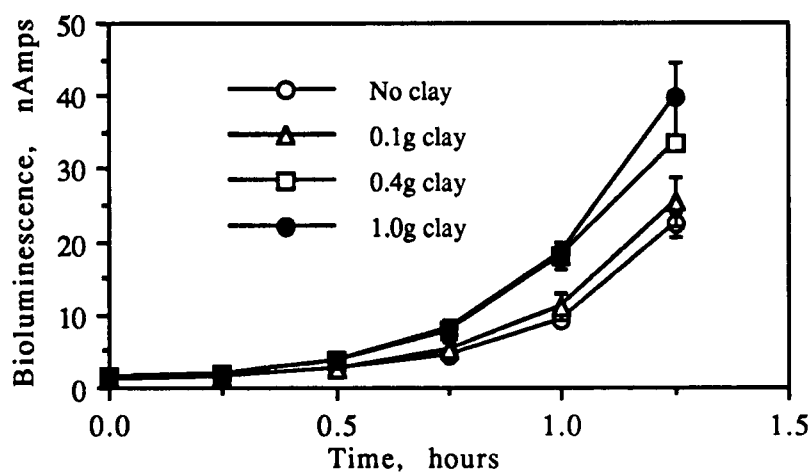


Figure 20. Bioluminescence response to naphthalene by strain HK44 immobilized in alginate beads (second test). Each vial contained 4 mL SSM with 2.5 mg L^{-1} naphthalene and 1.5 g of freshly made alginate beads with a total of approximately 10^9 cells.

use of immobilized cells introduced many more questions about the system: do all the cells within the bead have the same access to the substrate; how do the beads affect light output; can light be detected from all the cells in the bead, or only those near the surface; etc. However, a comparison of the bioluminescence patterns for suspended cells and immobilized cells suggests that naphthalene uptake, as evidenced by light output, was altered by elimination of contact between the organisms and the clay.

8.0 DISCUSSION

The role played by clay particles in microbial suspensions has been studied from the standpoint of effects on both the concentration of naphthalene outside the cell, and the rate of uptake into the cell as measured by mineralization and bioluminescence response. Adsorption, external mass transfer limitations, and intraparticle diffusion were all considered as factors that would affect the extracellular concentration. In a progressive examination, all three of these potentially rate-limiting mechanisms were systematically eliminated. Adsorption of naphthalene onto the clay surfaces was found to be negligible. Increasing the mixing speed of the suspensions, which would serve to decrease the thickness of a mass transfer-limiting boundary layer, caused no change in the typical light output pattern. Application of naphthalene immediately before the assay did not result in increased light levels when compared with samples in which a 24 hour contact period had been allowed for diffusion of naphthalene into intraparticle micropores. In each experiment, light output was observed to decrease with increasing clay concentration, and there was virtually no change in light output for the same clay concentration under different conditions of mixing or substrate application. Bioluminescence responses of organisms provided with decreasing amounts of naphthalene in the absence of clay have indicated that cells exposed to lower concentrations of naphthalene produce less light (Figure 5). Therefore, it would appear that increasing the clay content has the effect of reducing the concentration of naphthalene which is taken

into the cell, and that, based on experimental results, this reduction is not caused by adsorption, boundary layer diffusion, or intraparticle diffusion. A change in the inverse relationship between clay concentration and light output was not observed until direct contact between the clay and the organisms was eliminated by immobilizing the cells in alginate. The light response from the immobilized cells was also further evidence that the effect was not due to a clay-substrate interaction or to interference of clay with the detection method. However, in the absence of any positive identification of direct cell-clay contact, a challenge is presented to create a conceptual model that will reasonably and satisfactorily describe the interactions that produced the results of this study.

The most frequent and consistent observation was that in both the mineralization and the bioluminescence assays an increase in clay quantity had the effect of reducing the parameter that was being measured by the assay, either CO₂ or light production. A closer look at the data however, suggests that although the same relative effect among the different clay concentrations occurred in both assays, the pattern of the light response did not duplicate the mineralization response for any one clay quantity. When compared with the graphs of the mineralization data, it can be seen that the bioluminescence data do not always appear to reflect metabolic rate. For example, in the bioluminescence/mineralization correlation experiment (Figures 5 through 7), the mineralization rate seemed to remain fairly constant over the two hours of the experiment. The bioluminescence data however, showed a definite increase in light production in the second

hour. This rapid increase in light output was observed in many of the bioluminescence assays. In some cases, the level of light output increased ten-fold in less than two hours. A parallel increase was not observed in the mineralization assays. In fact, plots of the data for naphthalene mineralization in the presence of montmorillonite showed that the rate generally decreased with time (Figures 2 and 7A).

The observed rapid increase in light output, the inverse relationship between clay content and light or mineralization, and the suggestion of a direct clay-cell interaction all lead to the formation of a conceptual model which is based on three assumptions: (1) the light response consists of two separate phases, (2) uptake of naphthalene occurs by passive transport and is proportional to the concentration in solution, and (3) clay particles physically block uptake sites on the membrane or otherwise alter membrane permeability. The major portion of this discussion will present supporting arguments for these three assumptions.

8.1 Light Response Characteristics

Many of the bioluminescence experiments were designed to be completed within two hours of beginning the assay. In experiments of longer duration the peak light response values were so large that when the results were plotted, the initial differences among the various clay conditions were undetectable unless plotted logarithmically. The peak response was also not reproducible, although it was generally observed that increasing the clay content delayed the peak light response. The actual magnitude of the peak could not be recorded with confidence

without continuous monitoring of each sample, due to the rate of change in the response during the rapid increase. The intensity and duration of the peak response, along with the lack of any such rapid increase in the mineralization rate, suggest that the increase may be unrelated to metabolic rate or substrate concentration. The complete light response can best be described as having two phases. In the first phase, light output appears to be proportional to the rate at which naphthalene is metabolized, and is probably related to the initial rate of enzyme production. After a certain recognition period, the second phase occurs in which it appears that light output becomes independent of the rate of uptake. The existence of two phases is consistent with the manner in which the bioluminescence and biodegradation mechanisms are regulated by the cell. According to DiGrazia (1991), transcription and translation of the enzymes required for both processes are linked together, after which the two pathways are completely independent except for energy and oxygen requirements.

The suggestion that the light response occurs in two phases has certain implications with respect to the role of clay particles. For the initial light output to be inversely proportional to the clay content, an increase in the quantity of clay in suspension would have to cause an increasing interference with the uptake of naphthalene. In the second phase, although the effect of the clay is probably still present, the light response is no longer dependent on the rate of uptake. In addition to the observations made in this research, two findings by Burlage and coworkers (1990) and DiGrazia (1991) support the idea that after an initial exposure period, the level of light produced is independent of the

concentration of naphthalene in solution: (1) peak light levels were not observed until several hours after exposure to naphthalene for both growing and non-growing cells, and (2) high levels of light output were observed even at low concentrations of naphthalene. There is some evidence to support the claim made by Burlage, that bioluminescence is greatest at a slow growth rate. A rapid increase in the light response was observed to occur in those experiments using cells prepared by Growth Procedure I, which were determined by plate counts to be non-growing. However, even higher light levels were recorded in the experiment in which optical density was recorded, when the cells were observed to be in log growth. These high levels also occurred at much lower concentrations of naphthalene, again suggesting a peak light response phase that is independent of both growth and naphthalene concentration.

8.2 Uptake of Naphthalene

In discussing the different phases of light response, references to the rate of uptake were used interchangeably with concentration of naphthalene. The validity of this interchange has its basis in the second assumption - that uptake of naphthalene occurs by passive transport and is proportional to the concentration in solution. As discussed previously (Section 2.1.1), due to its hydrophobic nature, naphthalene is believed to pass into the cell by a passive transport mechanism, independent of any energy producing process. Passive transport of a substrate into the cell can occur either by passive or facilitated diffusion. Passive or simple diffusion is the non-specific

penetration of a substance into the cell, typically at low rates, governed by molecular size and lipophilic properties (Schlegel, 1985). In facilitated diffusion, transport of a substance into the cell is mediated by a substrate-specific permease, at a rate governed by the substrate concentration in the medium. Both passive and facilitated diffusion are independent of metabolic energy and the substrate cannot accumulate inside the cell against a concentration gradient. Button (1983) proposed that the passive partitioning of lipophilic substrates was capable of producing high concentrations of the substrate near membrane-bound enzymes from small concentrations in the environment. Based on this supposition, it would be reasonable to assume that passive diffusion of naphthalene would result in relatively constant concentrations within the cell, and little variation in the light response, even over a fairly broad range of extracellular concentrations. In this study however, light output was observed to decrease with decreasing naphthalene concentration in the absence of clay in what is being assumed as the first phase of light production. This observation suggests that uptake of naphthalene may occur by facilitated diffusion, however there is insufficient evidence to positively identify the mechanism. If a decrease in bioluminescence during the first phase of light production is seen as a response to a decreased concentration of naphthalene, then increasing the clay quantity can be viewed as having the same effect.

8.3 The Cell-Clay Interaction

The third assumption suggests that the mechanism by which the clay particles act to decrease the concentration of naphthalene "seen"

by the cells is an alteration in the rate of diffusion of naphthalene through the membrane due to direct contact between the cells and the clay particles. Whether uptake occurs by passive or facilitated diffusion, passage of naphthalene molecules through the membrane could be inhibited either by changes in membrane permeability, blockage of specific diffusion sites, or both. Because the inverse clay-light relationship was not affected by increased mixing or by altering the stability of the clay aggregates, a cell-clay association would appear to be some type of adhesion rather than a weak electrostatic or hydrodynamic association, although a sufficient accumulation of weak H bonds can result in an extremely strong interaction (Stotzky, 1986). The result of changing particle material also implicated cell-clay contact as the source of reduced light output. When sand particles were used instead of clay, the inhibitory effect of particle concentration on light output was not observed. Due to particle size differences, the same mass of clay presents many more particles and surface area for microbial contact than sand. The strongest evidence for a cell-clay interaction is the change in the light response observed when immobilized cells were used (Figures 19 and 20). Elimination of contact between the particles and the cells was the first experimental treatment that altered the typical light response pattern in the presence of clay.

Particulate blocking of substrate transport sites or alteration of membrane permeability as a result of surface charge interactions have been suggested by several researchers (Button, 1983; Jeffrey and Paul, 1986; 1984; Marshall, 1969). Hattori and Hattori (1987) reported an increase in microbial activity as measured by O₂ consumption after cells

that had been attached to ion exchange resin were released by shaking with a concentrated NaCl solution. One of the reasons proposed for the decreased activity of the attached cells was that cell surface area which takes part in substrate uptake was decreased by attachment. Kieft and Caldwell (1986) also stated that attached cells have a decreased surface area and thus cannot absorb or transport nutrients as rapidly as can unattached cells. Jannasch and Pritchard (1972) observed that the presence of inert particulate matter not only exerted a considerable influence on microbial activity, but that attachment of cells occurred earlier as the quantity of particles increased.

If a barrier exists between a portion or all of the membrane area and the bulk solution, oxygen uptake may be also be affected. Jannasch and Pritchard (1972) reported that the addition of particles caused clumps to form in the cell suspension leading to increased intra-aggregate oxygen consumption and the formation of anaerobic pockets in spite of high concentrations of dissolved oxygen in the surrounding water. Even under well-mixed conditions, localized depletion of oxygen is a likely cause for the decrease in light production per million organisms that was observed when the cell density was increased from 10^8 to 10^{10} cells per sample vial. As oxygen is one of the key requirements for the light producing pathway, its limitation is an immediate suspect when a reduction in bioluminescence is observed.

Several attempts have been made to examine cell-particle interactions microscopically. Because of the opaque and colloidal nature of clays, it is difficult to view undisturbed microhabitats microscopically for direct observation of clay-microbe associations.

Marshall (1969) indirectly demonstrated the presence of a clay coating around *Bacillus subtilis* organisms by decreased intensity of the bacterial image under fluorescence microscopy. In the same study, electron micrographs appeared to confirm the presence of a montmorillonite clay envelope adsorbed around *Rhizobium* isolates, however, it is unclear how the process of freeze-drying or surface preparation may have affected the samples (Figure 21). A similar clay envelope was observed by Roper and Marshall (1979) in association with *E. coli* (Figure 22). In another study, Marshall and coworkers (1971) observed adhesion of large and small bacterial rods in the absence of noticeable attachment structures, and suggested production of extracellular polymeric materials. Based on scanning electron microscopic photographs, Kieft and Caldwell (1986) estimated that 15 to 20% of the surface area of a *Vibrio* species could be lost owing to attachment. The specific ability of a *Pseudomonas* species to attach actively to suspended particles was observed by Jannasch and Pritchard (1972) although no morphological mechanism for attachment was detected. In observing the attachment of two strains of *Pseudomonas fluorescens* to glass cover slips, Fletcher (1988) also suggested that a cell-bound extracellular material might be involved in adhesion.

An attempt was made to view the clay-microbe suspensions typical of this research with an environmental scanning electron microscope (ESEM - ElectroScan, Wilmington, MA). The ESEM allows observation of a wet sample without prior surface preparation by causing the sample drop to condense directly on the mounting stand.



Figure 21. Electron micrographs from Marshall, K.C. 1969. *J. Gen. Microbiol.* **56**:301-306. (A) *R. trifolii* SU297B plus montmorillonite ($80 \mu\text{g mL}^{-1}$). Unshadowed; $\times 34,500$. (B) *R. trifolii* SU297B plus illite ($100 \mu\text{g mL}^{-1}$). Unshadowed; $\times 34,500$.



Figure 22. Electron micrograph from Roper and Marshall. 1979. *Microb. Ecol.* 4(4)279-289. *E. coli* coated with montmorillonite, shadowed; bar = 1 μ m.

Unfortunately, although the clay particles were quite distinct, there was not sufficient resolution to produce a clear image showing the position of the microorganisms with respect to the clay. This is typical of the problems encountered in studying the activity of microorganisms in clay- or soil-containing systems. The lack of adequate experimental techniques is a major limitation for conducting these studies.

8.4 The Conceptual Model

On the basis of the three assumptions discussed above, the conceptual model can be described. The microcosm is known to consist of an aqueous slurry containing separate and aggregated clay particles. The majority of the aggregates remain settled out even during mixing, but the smaller aggregates and single particles can become suspended with slight fluid motion. The aqueous solution is buffered and contains various inorganic salts at low concentrations. Naphthalene is the only carbon source. *Pseudomonas fluorescens* possess polar flagellae (Figure 23), and are able to move freely within the microcosm even in the absence of mechanical mixing (Schlegel, 1985). It is suggested that when the cells come in contact with the clay, sub-micron sized particles collect at the surface of the organisms, and organisms become attached to or embedded within larger aggregates. Attachment or adherence may result from an accumulation of H bonds between functional groups on the surfaces of the particles and the cells, or from an adhesive property of the cell membrane. Naphthalene molecules are assumed to be taken up into the cells either by hydrophobic partitioning or by facilitated diffusion. In the absence of clay, uptake of naphthalene is



Figure 23. Electron micrograph from Burgess et al. 1987. *Under the Microscope*, Cambridge University Press, Cambridge, p.94. *P. fluorescens*. TEM, surface replica, carbon + platinum coating; x 18,750.

believed to occur freely at a rate that is governed by its concentration in solution. In the presence of clay particles, some of the diffusion sites on the cell membrane may be blocked and the uptake rate decreased. It is suggested that the blocking of uptake sites on the membrane has an effect that is similar to decreasing the solution concentration of naphthalene. In a mineralization assay, the presence of clay particles results in a decrease in the rate of CO₂ production that is consistent with a decrease in the uptake rate. In a bioluminescence assay, the effect of the clay is initially seen as a decrease in light output, similar to that which occurs when the naphthalene concentration is decreased in the absence of clay. After a period of exposure however, the light output becomes independent of naphthalene concentration and a rapid increase in light output is observed regardless of clay quantity.

8.5 Implications for Practical Applications and Future Study

There is a good deal of interest in the use of bioluminescent reporter technology for predicting and monitoring microbial biodegradative processes such as treatment of contaminated soil or groundwater. The results of this research indicate that, as with any other technology, interpretation of the results is dependent upon recognition of the limitations of the assay. Although an attempt has been made to quantitatively correlate the light response with metabolic rate as measured by CO₂ production, bioluminescence remains a qualitative assay. In the case of strain HK-44, it appears that after a

certain period of exposure to naphthalene, the light response does not accurately reflect either the concentration of naphthalene, or the rate of uptake. Conclusions as to the effect that particles may have on the activity of this particular organism are therefore dependent on the length of the assay, and the time of the measurements. For example, in the five hour bioluminescence assay in which a cell density of 10^{10} was used, it was observed that peak levels of light were eventually achieved at all clay concentrations. It would be simple to interpret these results as an indication that the clay particles had no long-term effect on microbial activity. However, repeated examinations of the short term response all point to an inhibitory effect, and it is more reasonable to assume that the peak light response is an indication of insensitivity to the effect rather than that the effect was somehow reversed.

If these experiments had been part of a treatability study that was to form the basis for a full-scale soil remediation project, different approaches may have been planned depending on which type of experimental procedure was followed in the lab. The results of the mineralization assay alone may have indicated that the best approach would be to use a slurry-phase remediation process keeping the solids content below 20%. If the treatability experiment had been a 12 hour bioluminescence assay using cells grown overnight and washed prior to use, the conclusions may have been that a long lag time could be expected, but that high degradative rates could eventually be achieved regardless of the soil to liquid ratio. If the basis for the scale-up had been a series of two hour bioluminescence assays using cells in mid-log growth, it may have been decided that the soil would significantly

retard the remediation process, and a soil washing process followed by aqueous phase biotreatment may have been recommended.

There is always a temptation to generalize data that were obtained from a specific situation. As in other reductionistic studies, the results apply to an extremely narrow range of experimental conditions. The findings made in this study are limited to the uptake of one substrate by a pure culture of one specific organism in the presence of one soil constituent in a batch microcosm. As with any living, dynamic system, changes to or the addition of environmental variables can significantly alter the outcome of any experiment. That is not to say that certain fundamental principles do not remain constant, but rather that the expression of those principles may vary within different contexts. Bioluminescent reporter technology is an example of this. The principle for the technology is that when an organism possesses the *lux* genes fused to the promoter for a degradative pathway, the detection of bioluminescence will always indicate that the pathway is active at some level. However, the level of expression can be attributed to any number of variables, depending on the context of the measurement. For bioluminescence technology to develop into a quantitative rather than a qualitative assay, future research should focus on separating the principle from the context. It should be determined whether light output is in fact a two phase process, and if so, whether that process is unique to a specific gene fusion. If the two phase hypothesis is disproved, the reasons for the rapid increase in light production need to be examined. The relationship between bioluminescence and mineralization needs to be more fully characterized - a study which

would be both labor and material intensive. And finally, the matter that was at the heart of this research, more knowledge is needed regarding the physical interaction between microorganisms and clays. The development of new microscopic techniques such as the ESEM holds great promise for gaining greater insight into the behavior of microorganisms in soil habitats. After decades of indirect observations, a visual investigation may hold the key to many unanswered questions.

9.0 CONCLUSIONS

The results of this research confirmed previous observations regarding an inhibitory effect of clay on the degradation of naphthalene by suspended microorganisms. Identification of the mechanism for the inhibition remains elusive. Negative findings were made concerning possible rate-limitations due to adsorption, boundary layer mass transfer, and intraparticle diffusion. The inhibitory effect was not observed when direct clay-microbe contact was eliminated by using cells immobilized in alginate beads. On the basis of these observations as well as certain assumptions concerning the transport of naphthalene into the cell, a conceptual model has been proposed in which attachment of the cells to clay particles results in blockage of diffusion sites on the cell membrane and a decrease in the rate of naphthalene uptake. Direct observation by microscopy may be required in future research for positive confirmation of a cell-clay interaction.

The use of bioluminescent reporter technology provided the means to perform many experiments rapidly and inexpensively. However, a comparison of the results with those from mineralization assays was required for meaningful interpretation. Reproducibility of the results from bioluminescence experiments was highly dependent on the cell growth procedure. In addition, after approximately two hours of exposure of the cells to naphthalene, a rapid increase in light

output could be observed that appeared to be unrelated to the degradation rate. The cause of the rapid increase is unknown and requires further investigation.

Although the results of this research apply specifically only to a limited and highly controlled environment, the potential for clay particles to inhibit microbial activity has implications for possible approaches to the bioremediation of a whole soil. In particular, if bioluminescence assays are to be used in the future for treatability tests, it should be understood that cell preparation methods and solid to liquid ratios can have a substantial impact on the experimental results.

LIST OF REFERENCES

LIST OF REFERENCES

- Angle, J.S., S.P. McGrath, and R.L. Chaney. 1991. New culture medium containing ionic concentrations of nutrients similar to concentrations found in the soil solution. *Appl Environ. Microbiol.* **57**(12):3674-3676.
- Ball, W.P. 1989. Equilibrium sorption and diffusion rate studies with halogenated organic chemicals and sandy aquifer material. Ph.D. dissertation. Stanford University, CA.
- Banerjee, P., M.D. Piwoni, and K. Ebeid. 1985. Sorption of organic contaminants to a low carbon subsurface core. *Chemosphere* **14**(8):1057-1067.
- Beloin, R.M., J.L. Sinclair, and W.C. Ghiorse. 1988. Distribution and activity of microorganisms in subsurface sediments of a pristine study site in Oklahoma. *Microb. Ecol.* **16**:85-97.
- Bettmann, H. and H.J. Rehm. 1984. Degradation of phenol by polymer entrapped microorganisms. *Appl. Microbiol. Biotechnol.* **20**:285-290.
- Black, W.V., R.C. Ahlert, D.S. Kosson, and J.E. Brugger. 1991. Slurry-based biotreatment of contaminants sorbed onto soil constituents. In R.E. Hinchee and R.F. Olfenbuttel, (eds.), *On-Site Bioreclamation - Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Boston. pp. 408-422.
- Bohn, H.L., B.L. McNeal, and G.A. O'Connor. 1985. *Soil Chemistry*; 2nd ed, John Wiley and Sons, New York. pp.135-150.
- Brusseau, M.L., R.E. Jessup, and P.S.C. Rao. 1991. Nonequilibrium sorption of organic chemicals: Elucidation of rate-limiting processes. *Environ. Sci. Technol.* **25**(1):134-142.
- Burlage, R.S., G.S. Sayler, and F. Larimer. 1990. Monitoring of naphthalene catabolism by bioluminescence with *nah-lux* transcriptional fusions. *J. Bacteriol.* **172**(9):4749-4757.
- Button, D.K. 1983. Differences between the kinetics of nutrient uptake by micro-organisms, growth and enzyme kinetics. *Trends Biochem. Sci.* April, 1983:121-124.
- Cerniglia, C.E. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. *Adv. Appl. Microbiol.* **30**:31-71.
- Dhawan, S., L.T. Fan, L.E. Erickson, and P. Tuitemwong. 1991. Modeling, analysis, and simulation of bioremediation of soil aggregates. *Environ. Prog.* **10**(4):251-260.

DiGrazia, P.M. 1991. Microbial systems analysis of naphthalene degradation in a continuous flow soil slurry reactor. Ph.D. dissertation. University of Tennessee, Knoxville, TN.

DiGrazia, P.M., J.W. Blackburn, P.R. Bienkowski, B. Hilton, G.D. Reed, J.M.H. King, and G.S. Sayler. 1990. Development of a systems analysis approach for resolving the structure of biodegrading soil systems. *Appl. Biochem. Biotech.* **24/25**:237-252.

Edwards, N.T. 1983. Polycyclic aromatic hydrocarbons (PAH's) in the terrestrial environment - a review. *J. Environ. Qual.* **12**(4):427-441.

Estes, T.J., R.V. Shah, and V.L. Vilker. 1988. Adsorption of low molecular weight halocarbons by montmorillonite. *Environ. Sci. Technol.* **22**:377-381.

Evolutionary Software, Inc. 1991. *E4 User's Guide, Version 91.1*. Evolutionary Software, Inc., Knoxville, Tennessee. 59 pp.

Fletcher, M. 1988. Attachment of *Pseudomonas fluorescens* to glass and the influence of electrolytes on bacterium-substratum separation distance. *J. Bacteriol.* **170**(5):2027-2030

Gee, G.W. and J.W. Bauder. 1986. Particle-size analysis. In A. Klute, (ed.), *Methods of Soil Analysis Part 1. Physical and Mineralogical Methods*, 2nd ed. Agron. Monogr. 9. American Society of Agronomy, Madison, WI. pp.383-411.

Gibson, D.T. 1971. The microbial oxidation of aromatic hydrocarbons. *CRC Crit. Rev. Microbiol.* **1**:199-223.

Gordon, A.S., S.M. Gerchakov, and F.J. Millero. 1983. Effects of inorganic particles on metabolism by a periphytic marine bacterium. *Appl. Environ. Microbiol.* **45**(2):411-417.

Guerin, W.F. and S.A. Boyd. 1992. Differential bioavailability of soil-sorbed naphthalene to two bacterial species. *Appl. Environ. Microbiol.* **58**(4):1142-1152.

Harriott, P. 1962. Mass transfer to particles: Part I. Suspended in agitated tanks. *A.I.Ch.E. Journal.* **8**(1):93-101.

Harter, R.D. 1977. Reactions of minerals with organic compounds in the soil. In J.B. Dixon and S.B. Weed, (eds.), *Minerals in Soil Environments*, 1st Ed. SSSA Book Series Number 1. Soil Science Society of America, Madison, WI. pp. 709-739.

Hassett, J.J., and W.L. Banwart. 1989. The sorption of nonpolar organics by soils and sediments. In B.L. Sawhney and K. Brown, (eds.), *Reactions and Movement of Organic Chemicals in Soils*. SSSA Special Publication No. 22. Soil Science Society of America, Madison, WI. pp. 31-44.

- Hassett, J.J., W.L. Banwart, S.G. Wood, and J.C. Means. 1981. Sorption of α -naphthol: implications concerning the limits of hydrophobic sorption. *Soil Sci. Soc. Am. J.* 45:38-42.
- Hattori, R. and T. Hattori. 1987. Interaction of microorganisms with a charged surface - a model experiment. *Rep. Inst. Agr. Res. Tohoku Univ.* 36:21-67.
- Jannasch, H.W. and P.H. Pritchard. 1972. The role of inert particulate matter in the activity of aquatic microorganisms. *Mem. Ist. Ital. Idrobiol.* 29 Suppl.:289-308.
- Jeffrey, W.H., and J.H. Paul. 1986. Activity of an attached and free-living *Vibrio* sp. as measured by thymidine incorporation, *p*-iodonitrotetrazolium reduction, and ATP/ADP ratios. *Appl. Environ. Microbiol.* 51:150-156.
- Jury, W.A., W.F. Spencer, and W.J. Farmer. 1983a. Use of models for assessing relative volatility, mobility, and persistence of pesticides and other trace organics in soil systems. In J. Saxena (ed.), *Hazard Assessment of Chemicals - Current Developments*. Vol. 2. Academic Press, New York. pp.1-43.
- Jury, W.A., W.F. Spencer, and W.J. Farmer. 1983b. Behavior assessment model for trace organics in soil: I. Model description. *J. Environ. Qual.* 12(4):558-564.
- Karickhoff, S.W., D.S. Brown, and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. *Water Res.* 13:241-248.
- Karickhoff, S.W. and K.R. Morris. 1985. Sorption dynamics of hydrophobic pollutants in sediment suspensions. *Environ. Toxicol. Chem.* 4:469-479.
- Kieft, T.L. and D.E. Caldwell. 1984. Chemostat and in-situ colonization kinetics of *Thermothrix thiopara* on calcite and pyrite surfaces. *Geomicrobiol. J.* 3(3):217-229.
- King, J.M.H., P.M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G.S. Sayler. 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science.* 249:778-781.
- Korde, M. 1991. Effect of soil composition and contamination on naphthalene mineralization. M.S. Thesis. University of Tennessee, Knoxville.
- Kunc, F. and G. Stotzky. 1974. Effect of clay minerals on heterotrophic microbial activity in soil. *Soil Science.* 118(3):186-195.
- Laha, S. and R.G. Luthy. 1991. Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. *Environ. Sci. Technol.* 25(1):1920-1930.

- Lawrence, J.R., P.F. Delaquis, D.R. Korber, and D.E. Caldwell. 1987. Behavior of *Pseudomonas fluorescens* within the hydrodynamic boundary layers of surface microenvironments. *Microb. Ecol.* **14**:1-14.
- Letterman, R.D., J.E. Quon, and R.S. Gemmell. 1974. Film transport coefficient in agitated suspensions of activated carbon. *J. Water Pol. Contr. Fed.* **46**(11):2536 - 2546.
- Marshall, K.C. 1968. Interaction between colloidal montmorillonite and cells of *Rhizobium* species with different ionogenic surfaces. *Biochim. Biophys. Acta.* **156**:179-186.
- Marshall, K.C. 1969. Studies by microelectrophoretic and microscopic techniques of the sorption of illite and montmorillonite to rhizobia. *J. Gen. Microbiol.* **56**:301-306.
- Marshall, K.C. 1980. Adsorption of microorganisms to soils and sediments. In G. Bitton and K.C. Marshall, (eds.), *Adsorption of Microorganisms to Surfaces*. John Wiley and Sons, New York. pp.317-329.
- Marshall, K.C., R. Stout, and R. Mitchell. 1971. Selective sorption of bacteria from seawater. *Can. J. Microbiol.* **17**:1413-1416.
- Marshman, N.A. and K.C. Marshall. 1981a. Some effects of montmorillonite on the growth of mixed microbial cultures. *Soil Biol. Biochem.* **13**:135-141.
- Marshman, N.A. and K.C. Marshall. 1981b. Bacterial growth on proteins in the presence of clay minerals. *Soil Biol. Biochem.* **13**:127-134.
- Means, J.C., S.G. Wood, J.J. Hassett, and W.L. Banwart. 1980. Sorption of polynuclear aromatic hydrocarbons by sediments and soils. *Environ. Sci. Technol.* **14**(12):1524-1528.
- Mihelcic, J.R. and R.G. Luthy. 1991. Sorption and microbial degradation of naphthalene in soil-water suspensions under denitrification conditions. *Environ. Sci. Technol.* **25**(1):169-177.
- Miller, M.E. and M. Alexander. 1991. Kinetics of bacterial degradation of benzylamine in a montmorillonite suspension. *Environ. Sci. Technol.* **25**(2):240-245.
- Ngian, K.F., S.H. Lin, and W.R.B. Martin. (1977). Effect of mass transfer resistance on the Lineweaver-Burk plots for flocculating microorganisms. *Biotechnol. Bioeng.* **19**:1773-1784.
- Ogram, A.V., R.E. Jessup, L.T. Ou, and P.S.C. Rao. 1985. Effects of sorption on biological degradation rates of (2,4-dichlorophenoxy)acetic acid in soils. *Appl. Environ. Microbiol.* **49**(3):582-587.
- Olness, A. and C.E. Clapp. 1972. Microbial degradation of a montmorillonite-dextran complex. *Soil Sci. Soc. Amer. Proc.* **36**:179-181.

- O'Reilly, K.T. and R.L. Crawford. 1989. Kinetics of *p*-cresol degradation by an immobilized *Pseudomonas* sp. *Appl. Environ. Microbiol.* **55**(4):866-870.
- Pignatello, J.J. 1989. Sorption dynamics of organic compounds in soils and sediments. In B.L. Sawhney and K. Brown, (eds.), *Reactions and Movement of Organic Chemicals in Soils*. SSSA Special Publication No. 22. Soil Science Society of America, Madison, WI. pp. 45-80.
- Rijnaarts, H.H.M., A. Bachmann, J.C. Jumelet, and A.J.B. Zehnder. 1990. Effect of desorption and intraparticle mass transfer on the biomineralization of α -hexachlorocyclohexane in a contaminated calcareous soil. *Environ. Sci. Technol.* **24**(9):1349-1354.
- Robinson, K.G., W.S. Farmer, and J.T. Novak. 1990. Availability of sorbed toluene in soils for biodegradation by acclimated bacteria. *Wat. Res.* **24**(3):345-350.
- Rochkind, M.L., J.W. Blackburn and G.S. Sayler. 1986. *Microbial decomposition of chlorinated aromatic compounds*. EPA/600/2-86/090. U.S. EPA, Hazardous Waste Engineering Research Laboratory, Cincinnati, OH.
- Rogers, R.D., J.C. McFarlane, and A.J. Cross. 1980. Adsorption and desorption of benzene in two soils and montmorillonite clay. *Environ. Sci. Technol.* **14**(4):457-460.
- Roper, M.M. and K.C. Marshall. 1979. Effects of a clay mineral on microbial predation and parasitism of *Escherichia coli*. *Microb. Ecol.* **4**(4):279-289.
- Santoro, T. and G. Stotzky. 1968. Sorption between microorganisms and clay minerals as determined by the electrical sensing zone particle analyzer. *Can. J. Microbiol.* **14**:299-307.
- Sayler, G.S. and R. Fox. 1991. Environmental biotechnology: Perceptions, reality, and applications. In Sayler et al (eds.), *Environmental Biotechnology for Waste Treatment*. Plenum Press, New York. pp.1-13.
- Schell, M.A. 1985. Transcriptional control of the *nah* and *sal* hydrocarbon-degradation operons by the *nahR* gene product. *Gene* **36**:301-309.
- Schlegel, H.G. 1985. *General Microbiology*, 6th ed. Cambridge University Press, Cambridge. 587 pp.
- Schnitzer, M. and S.U. Khan. 1972. *Humic Substances in the Environment*. Marcel Dekker Inc., New York, 327 pp.
- Scow, K.M. and J. Hutson. 1992. Effect of diffusion and sorption on the kinetics of biodegradation: theoretical considerations. *Soil Sci. Soc. Am. J.* **56**(1):119-127.
- Scow, K.M., S. Simkins, and M. Alexander. 1986. Kinetics of mineralization of organic compounds at low concentrations in soil. *Appl. Environ. Microbiol.* **51**(5):1028-1035.

- Sherwood, T.K. and P.L.T. Brian. 1968. Heat and mass transfer to particles suspended in agitated liquids. Office of Saline Water, U.S. Dept. of Int., Research and Development Progress Rept. No. 334.
- Sinclair, J.L. and W.C. Ghiorse. 1989. Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. *Geomicrobiol. J.* 7:15-31.
- Skinner, J.H., G.G. Ondich, T.L. Baugh. 1991. U.S. EPA bioremediation research programs. In R.E. Hinchey and R.F. Olfenbuttel, (eds.), *On-Site Bioreclamation - Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Boston. pp. 1-15.
- Stauffer, T.B. and W.G. MacIntyre. 1986. Sorption of low-polarity organic compounds on oxide minerals and aquifer material. *Environ. Toxicol. Chem.* 5:949-955.
- Stauffer, T.B., W.G. MacIntyre, and D.C. Wickman. 1989. Sorption of nonpolar organic chemicals on low-carbon-content aquifer materials. *Environ. Toxicol. Chem.* 8:845-852.
- Steinberg, S.M., J.J. Pignatello, and B.L. Sawhney. 1987. Persistence of 1,2-dibromoethane in soils: entrapment in intraparticle micropores. *Environ. Sci. Technol.* 21(12):1201-1208.
- Stotzky, G. 1966a. Influence of clay minerals on microorganisms III. Effect of particle size, cation exchange capacity, and surface area on bacteria. *Can. J. Microbiol.* 12:1235-1246.
- Stotzky, G. 1966b. Influence of clay minerals on microorganisms II. Effect of various clay species, homoionic clays, and other particles on bacteria. *Can. J. Microbiol.* 12:831-848.
- Stotzky, G. 1972. Activity, ecology, and population dynamics of microorganisms in soil. *Critical Rev. Microbiol.* 2:59-137.
- Stotzky, G. 1985. Mechanism of adhesion to clays, with reference to soil systems. In D.C. Savage and M. Fletcher, (eds.), *Bacterial Adhesion - Mechanisms and Physiological Significance*. Plenum Press, New York. pp.195-253.
- Stotzky, G. 1986. Influence of soil mineral colloids on metabolic processes, growth, adhesion, and ecology of microbes and viruses. In P.M. Huang and M. Schnitzer, (eds.), *Interactions of Soil Minerals with Natural Organics and Microbes*. SSSA Spec. Publ. 17. Soil Science Society of America, Madison, WI. pp. 305-428.
- Stotzky, G. and L.T. Rem. 1966. Influence of clay minerals on microorganisms I. Montmorillonite and kaolinite on bacteria. *Can. J. Microbiol.* 12:547-563.

- Thibodeaux, L.J. 1979. *Chemodynamics - Environmental Movement of Chemicals in Air, Water, and Soil*. John Wiley & Sons, New York. 501 pp.
- USEPA. 1980. *Carbon Adsorption Isotherms for Toxic Organics*. EPA-600/8-80-023. April 1980. Municipal Environmental Research Laboratory, Cincinnati, Ohio. pp. 318-319.
- van Loosdrecht, M.C.M., J. Lyklema, W. Norde, and A.J.B. Zehnder. 1989. Bacterial adhesion: A physicochemical approach. *Microb. Ecol.* **17**:1-15.
- Vandenhove, H., R. Merckx, H. Wilmots, and K. Vlassak. 1991. Survival of *Pseudomonas fluorescens* inocula of different physiological stages in soil. *Soil. Biol. Biochem.* **23**(12):1133-1142.
- van Loosdrecht, M.C.M., J. Lyklema, W. Norde, and A.J.B. Zehnder. 1990. Influence of interfaces on microbial activity. *Microbiol. Rev.* **54**(1):75-87.
- van Olphen, H. and J.J. Fripiat. 1979. *Data Handbook for Clay Materials and other Non-Metallic Minerals*. Pergamon Press, Oxford. pp. 13-34.
- Verschueren, K. and M.J. Visschers. 1988. The bioavailability of chemicals in waste products and in polluted soil. *Toxicol. Environ. Chem.* **16**:245-258.
- Weber, W.J. Jr. 1972. *Physicochemical Processes for Water Quality Control*. Wiley-Interscience, New York. pp. 210-211.
- Wu, S. and P.M. Gschwend. 1986. Sorption kinetics of hydrophobic organic compounds to natural sediments and soils. *Environ. Sci. Technol.* **20**(7):717-725.
- Yang, P.Y. and M.L. Wang. 1990. Entrapment of microbial cells for wastewater treatment. In R.D. Tyagi and K. Vembu, (eds.), *Wastewater Treatment by Immobilized Cells*. CRC Press, Boca Raton. pp. 45-77.
- Yare, B.S. 1991. A comparison of soil-phase and slurry-phase bioremediation of PNA-containing soils. In R.E. Hinchey and R.F. Olfenbittel, (eds.), *On-Site Bioreclamation - Processes for Xenobiotic and Hydrocarbon Treatment*. Butterworth-Heinemann, Boston. pp. 173-187.

APPENDICES

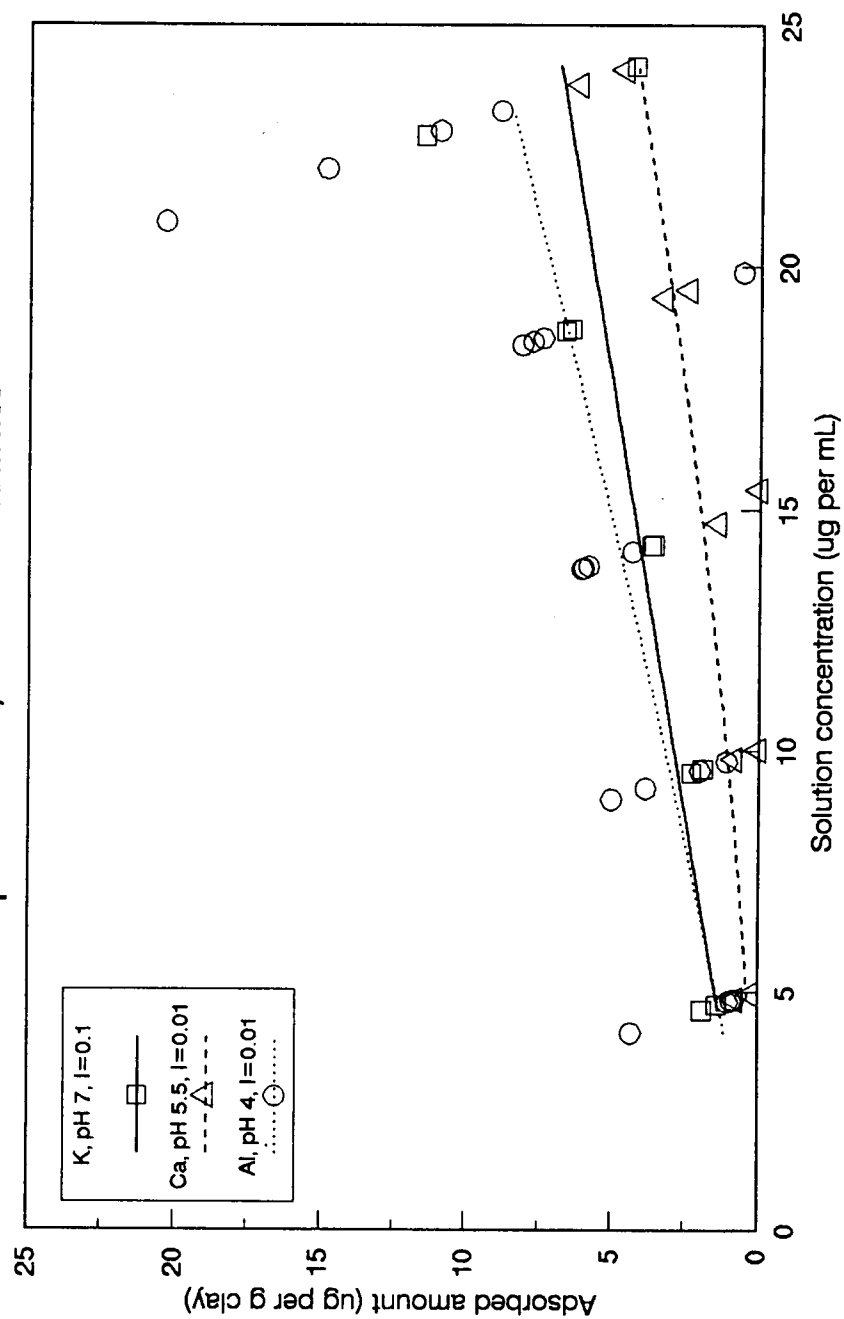
APPENDIX A

ADSORPTION ISOTHERMS: RAW DATA AND PLOTS

Adsorption Isotherm Data for KGa-2 Kaolinite

Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL		Naphthalene concentration (ug per 5 mL)	Naphthalene concentration (ug per mL)	Naphthalene adsorbed	
			solution after 24 hours	Percent DPM's in solution			adsorbed (by difference)	
							(ug per g clay)	
K, pH 7, I 0.1								
1a	0	0	0	0	0	0	0	
1b	0	0	0	0	0	0	0	
2a	25	27094	25060	0.9249	23.12	4.62	1.88	
2a	25	27094	25620	0.9456	23.64	4.73	1.36	
2b	50	54188	52160	0.9626	48.13	9.63	1.87	
3a	50	54188	51730	0.9546	47.73	9.55	2.27	
3b	75	81282	77320	0.9513	71.34	14.27	3.66	
4a	75	81282	77390	0.9521	71.41	14.28	3.59	
4b	100	108376	101190	0.9337	93.37	18.67	6.63	
5a	100	108376	101410	0.9357	93.57	18.71	6.43	
5b	125	135470	123040	0.9082	113.53	22.71	11.47	
6a	125	135470	130830	0.9657	120.72	24.14	4.28	
6b								
Ca, pH 5.5, I 0.01								
1a	0	0	0	0	0	0	0	
1b	0	0	0	0	0	0	0	
2a	25	27134	26330	0.9704	24.26	4.85	0.74	
2b	25	27134	26900	0.9914	24.78	4.96	0.22	
3a	50	54268	54450	1.0034	50.17	10.03	-0.17	
3b	50	54268	53400	0.9840	49.20	9.84	0.80	
4a	75	81402	79900	0.9815	73.62	14.72	1.38	
4b	75	81402	83650	1.0276	77.07	15.41	-2.07	
5a	100	108536	105010	0.9675	96.75	19.35	3.25	
5b	100	108536	105920	0.9759	97.59	19.52	2.41	
6a	125	135670	130630	0.9629	120.36	24.07	4.64	
6b	125	135670	128960	0.9505	118.82	23.76	6.18	
Al, pH 4, I 0.01								
1a	0	0	0	0	0	0	0	
1b	0	0	0	0	0	0	0	
2a	25	30744	29780	0.9686	24.22	4.84	0.78	
2b	25	30744	25450	0.8278	20.70	4.14	4.30	
3a	50	61488	60170	0.9786	48.93	9.79	1.07	
3b	50	61488	59050	0.9603	48.02	9.60	1.98	
4a	75	92232	84810	0.9195	68.96	13.79	6.04	
4b	75	92232	84870	0.9202	69.01	13.80	5.99	
5a	100	122976	113430	0.9224	92.24	18.45	7.76	
5b	100	122976	112990	0.9188	91.88	18.38	8.12	
6a	125	153720	140230	0.9122	114.03	22.81	10.97	
6b	125	153720	142800	0.9290	116.12	23.22	8.88	
Al, pH 4, I 0.01								
1a	0	0	0	0	0	0	0	
1b	0	0	0	0	0	0	0	
2a	25	30744	29660	0.9647	24.12	4.82	0.88	
2b	25	30744	29510	0.9599	24.00	4.80	1.00	
3a	50	61488	55330	0.8999	44.99	9.00	5.01	
3b	50	61488	56780	0.9234	46.17	9.23	3.83	
4a	75	92232	85090	0.9226	69.19	13.84	5.81	
4b	75	92232	86930	0.9425	70.69	14.14	4.31	
5a	100	122976	122270	0.9943	99.43	19.89	0.57	
5b	100	122976	113880	0.9260	92.60	18.52	7.40	
6a	125	153720	128670	0.8370	104.63	20.93	20.37	
6b	125	153720	135440	0.8811	110.14	22.03	14.86	

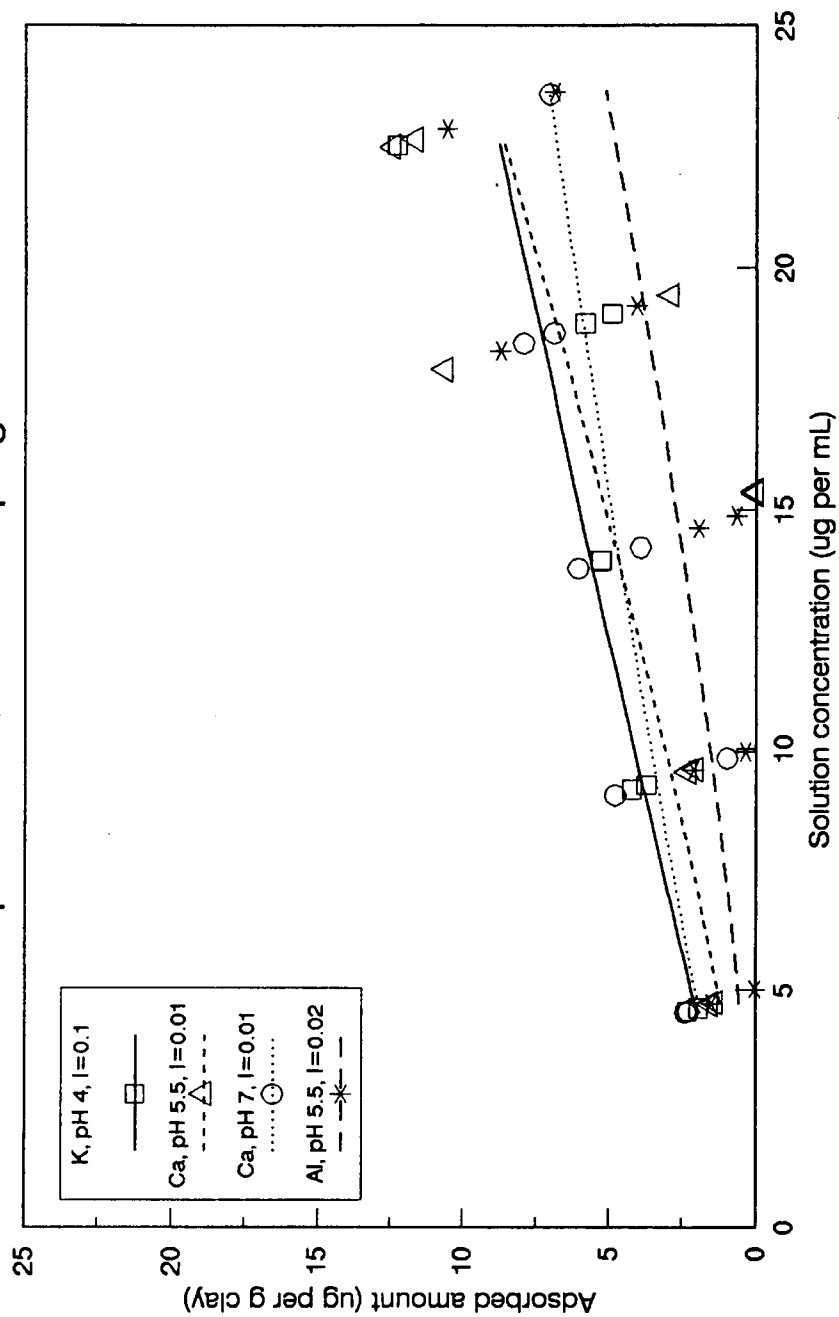
Adsorption Isotherm Naphthalene/KGa-2 Kaolinite



Adsorption Isotherm Data for PFI-1 Attapulgite

Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL solution after 24 hours	Percent DPM's in solution	Naphthalene concentration (ug per 5 mL)	Naphthalene concentration (ug per mL)	Naphthalene adsorbed (by difference) (ug per g clay)
K, pH 4, I 0.1							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	30120	27790	0.9226	23.07	4.61	1.93
2b	25	30120	27350	0.9080	22.70	4.54	2.30
3a	50	60240	55160	0.9157	45.78	9.16	4.22
3b	50	60240	55770	0.9258	46.29	9.26	3.71
4a	75	90360	84040	0.9301	69.75	13.95	5.25
4b	75	90360	83990	0.9295	69.71	13.94	5.29
5a	100	120480	114560	0.9509	95.09	19.02	4.91
5b	100	120480	113440	0.9416	94.16	18.83	5.84
6a	125	150600	135820	0.9019	112.73	22.55	12.27
6b	125	150600	135830	0.9019	112.74	22.55	12.26
Ca, pH 5.5, I 0.01							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	29806	28190	0.9458	23.64	4.73	1.36
2b	25	29806	28010	0.9397	23.49	4.70	1.51
3a	50	59612	56880	0.9542	47.71	9.54	2.29
3b	50	59612	57190	0.9594	47.97	9.59	2.03
4a	75	89418	91370	1.0218	76.64	15.33	-1.64
4b	75	89418	91660	1.0251	76.88	15.38	-1.88
5a	100	119224	115780	0.9711	97.11	19.42	2.89
5b	100	119224	106550	0.8937	89.37	17.87	10.63
6a	125	149030	135140	0.9068	113.35	22.67	11.65
6b	125	149030	134220	0.9006	112.58	22.52	12.42
Ca, pH 7, I 0.01							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	28638	25940	0.9058	22.64	4.53	2.36
2b	25	28638	26050	0.9096	22.74	4.55	2.26
3a	50	57276	56180	0.9809	49.04	9.81	0.96
3b	50	57276	51800	0.9044	45.22	9.04	4.78
4a	75	85914	78970	0.9192	68.94	13.79	6.06
4b	75	85914	81420	0.9477	71.08	14.22	3.92
5a	100	114552	106660	0.9311	93.11	18.62	6.89
5b	100	114552	105460	0.9206	92.06	18.41	7.94
6a	125	143190	135110	0.9436	117.95	23.59	7.05
6b	125	143190	110900	0.7745	96.81	19.36	28.19
Al, pH 5.5, I 0.02							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	27920	27980	1.0021	25.05	5.01	-0.05
2b	25	27920	26270	0.9409	23.52	4.70	1.48
3a	50	55840	53500	0.9581	47.90	9.58	2.10
3b	50	55840	55480	0.9936	49.68	9.94	0.32
4a	75	83760	81610	0.9743	73.07	14.61	1.93
4b	75	83760	83060	0.9916	74.37	14.87	0.63
5a	100	111680	107150	0.9594	95.94	19.19	4.06
5b	100	111680	101940	0.9128	91.28	18.26	8.72
6a	125	139600	131930	0.9451	118.13	23.63	6.87
6b	125	139600	127820	0.9156	114.45	22.89	10.55

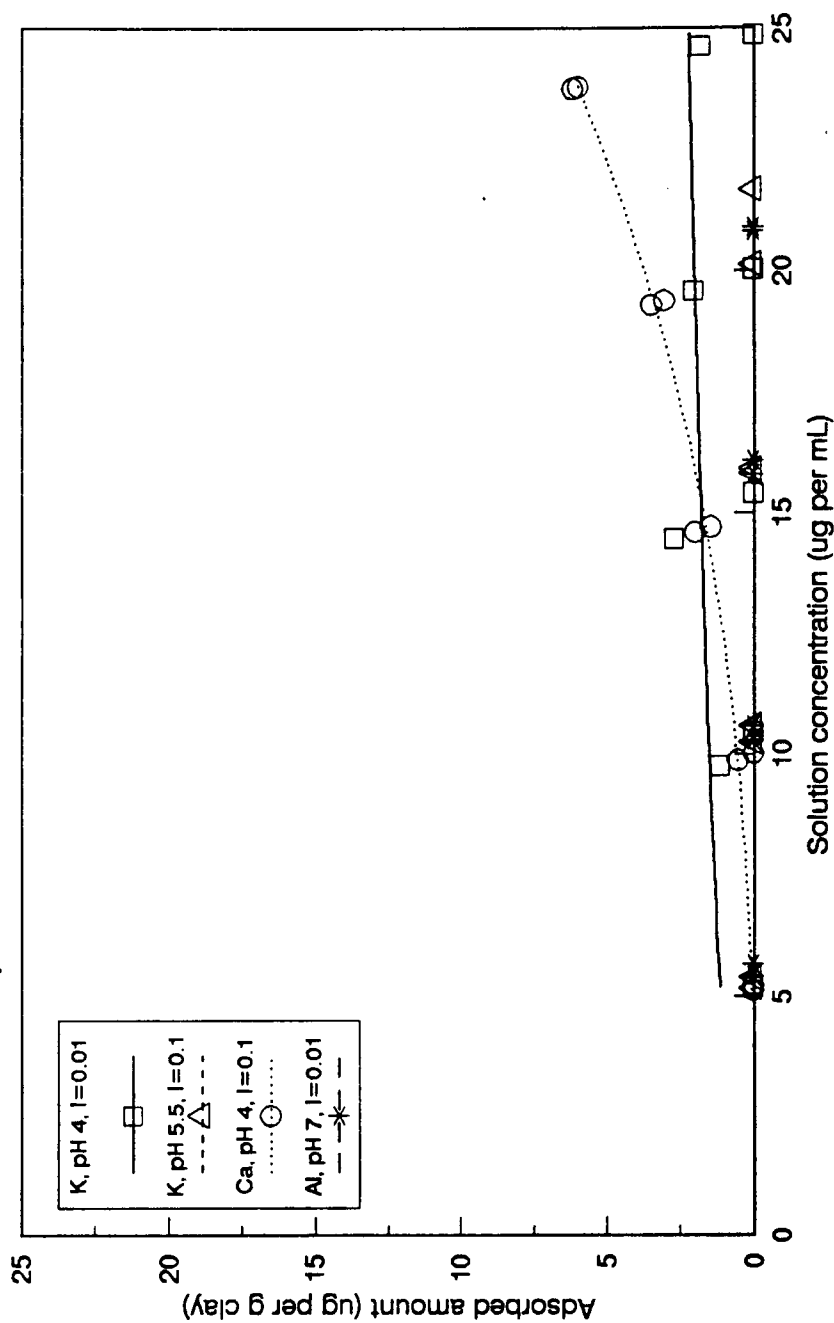
Adsorption Isotherm Naphthalene/PFI-1 Attapulгите



Adsorption Isotherm Data for SAZ-1 Montmorillonite

Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL		Percent DPM's in solution	Naphthalene concentration (ug per 5 mL)	Naphthalene concentration (ug perm L)	Naphthalene
			solution after 24 hours					adsorbed (by difference) (ug per g clay)
K, pH 4, I 0.01								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	36094	37390	1.0359	25.90	5.18	-0.90	
2b	25	36094	37860	1.0489	26.22	5.24	-1.22	
3a	50	72188	75540	1.0464	52.32	10.46	-2.32	
3b	50	72188	70500	0.9766	48.83	9.77	1.17	
4a	75	108282	111220	1.0271	77.03	15.41	-2.03	
4b	75	108282	104360	0.9638	72.28	14.46	2.72	
5a	100	144376	144630	1.0018	100.18	20.04	-0.18	
5b	100	144376	141420	0.9795	97.95	19.59	2.05	
6a	125	180470	179550	0.9949	124.36	24.87	0.64	
6b	125	180470	177860	0.9855	123.19	24.64	1.81	
K, pH 5.5, I 0.1								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	33978	36630	1.0781	26.95	5.39	-1.95	
2b	25	33978	35120	1.0336	25.84	5.17	-0.84	
3a	50	67956	69760	1.0265	51.33	10.27	-1.33	
3b	50	67956	72020	1.0598	52.99	10.60	-2.99	
4a	75	101934	108090	1.0604	79.53	15.91	-4.53	
4b	75	101934	107520	1.0548	79.11	15.82	-4.11	
5a	100	135912	147350	1.0842	108.42	21.68	-8.42	
5b	100	135912	136890	1.0072	100.72	20.14	-0.72	
6a	125	169890	175220	1.0314	128.92	25.78	-3.92	
6b	125	169890	176310	1.0378	129.72	25.94	-4.72	
Ca, pH 4, I 0.1								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	37994	38910	1.0241	25.60	5.12	-0.60	
2b	25	37994	39810	1.0478	26.19	5.24	-1.19	
3a	50	75988	75170	0.9892	49.46	9.89	0.54	
3b	50	75988	76190	1.0027	50.13	10.03	-0.13	
4a	75	113982	110940	0.9733	73.00	14.60	2.00	
4b	75	113982	111770	0.9806	73.54	14.71	1.46	
5a	100	151976	147360	0.9696	96.96	19.39	3.04	
5b	100	151976	146670	0.9651	96.51	19.30	3.49	
6a	125	189970	180590	0.9506	118.83	23.77	6.17	
6b	125	189970	180870	0.9521	119.01	23.80	5.99	
Al, pH 7, I 0.01								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	31348	34000	1.0846	27.11	5.42	-2.11	
2b	25	31348	35480	1.1318	28.30	5.66	-3.30	
3a	50	63696	67470	1.0593	52.96	10.59	-2.96	
3b	50	62696	64590	1.0302	51.51	10.30	-1.51	
4a	75	94044	100890	1.0728	80.46	16.09	-5.46	
4b	75	94044	99010	1.0528	78.96	15.79	-3.96	
5a	100	125392	131030	1.0450	104.50	20.90	-4.50	
5b	100	125392	130590	1.0415	104.15	20.83	-4.15	
6a	125	156740	160960	1.0269	128.37	25.67	-3.37	
6b	125	156740	162080	1.0341	129.26	25.85	-4.26	

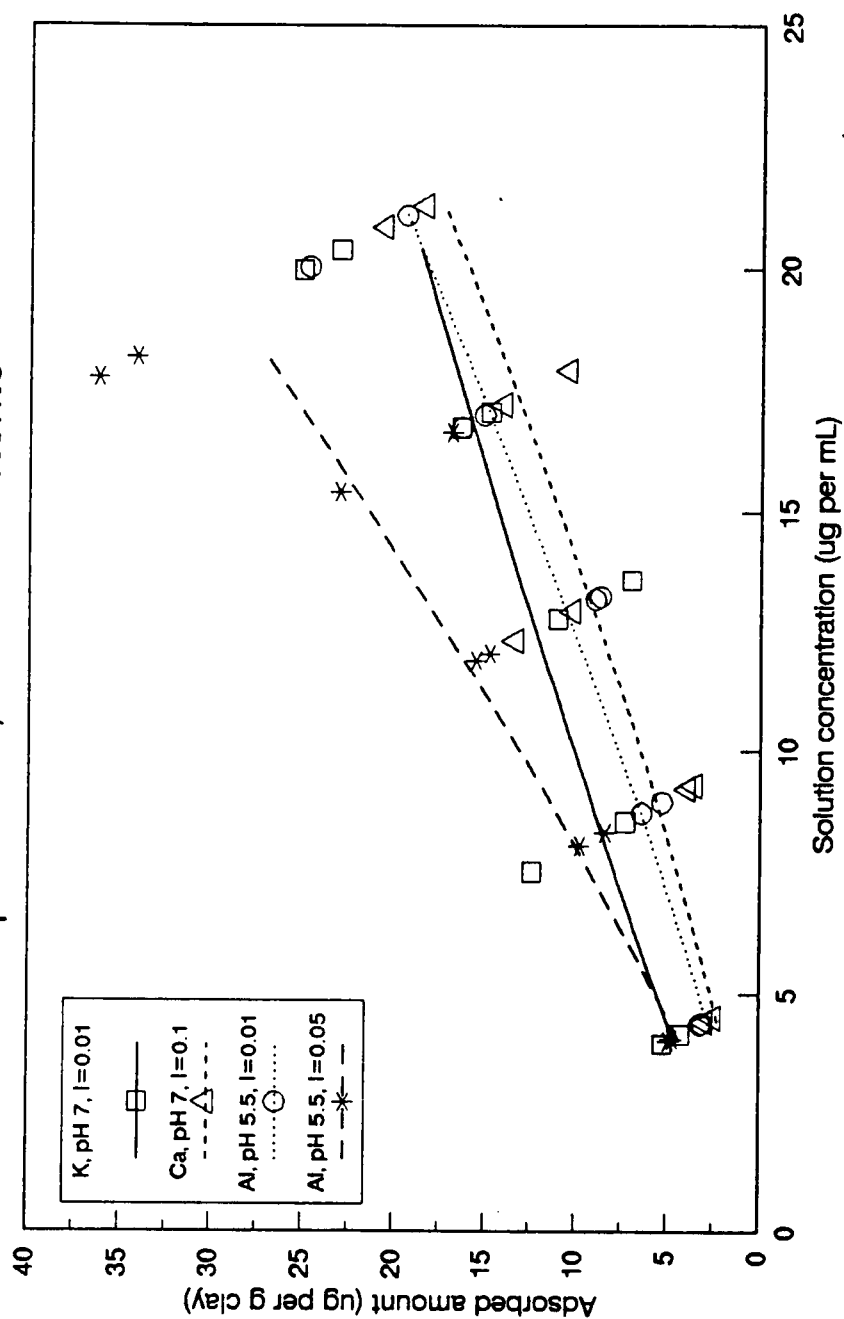
Adsorption Isotherm Naphthalene/SAZ-1 Montmorillonite



Adsorption Isotherm Data for SHCa-1 Hectorite

Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL		Percent DPM's in solution	Naphthalene concentration (ug per 5 mL)	Naphthalene concentration (ug per mL)	Naphthalene adsorbed (by difference) (ug per g clay)
			solution after 24 hours					
K, pH 7, I 0.01								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
1b	25	36048	28550	0.7920	19.80	3.96	5.20	4.26
2a	25	36048	29910	0.8297	20.74	4.15	4.26	4.26
2b	50	72096	61560	0.8539	42.69	8.54	7.31	7.31
3a	50	72096	54210	0.7519	37.60	7.52	12.40	12.40
3b	75	108144	98040	0.9066	67.99	13.60	7.01	7.01
4a	75	108144	92210	0.8527	63.95	12.79	11.05	11.05
4b	100	144192	120540	0.8360	83.60	16.72	16.40	16.40
5a	100	144192	122860	0.8521	85.21	17.04	14.79	14.79
5b	125	180240	144030	0.7991	99.89	19.98	25.11	25.11
6a	125	180240	146980	0.8155	101.93	20.39	23.07	23.07
6b								
Ca, pH 7, I 0.1								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	32516	28750	0.8842	22.10	4.42	2.90	2.90
2b	25	32516	29360	0.9029	22.57	4.51	2.43	2.43
3a	50	65032	60440	0.9294	46.47	9.29	3.53	3.53
3b	50	65032	59950	0.9219	46.09	9.22	3.91	3.91
4a	75	97548	84250	0.8637	64.78	12.96	10.22	10.22
4b	75	97548	80160	0.8217	61.63	12.33	13.37	13.37
5a	100	130064	116410	0.8950	89.50	17.90	10.50	10.50
5b	100	130064	111760	0.8593	85.93	17.19	14.07	14.07
6a	125	162580	135730	0.8349	104.36	20.87	20.64	20.64
6b	125	162580	138590	0.8524	106.56	21.31	18.44	18.44
Al, pH 5.5, I 0.01								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	33278	29240	0.8787	21.97	4.39	3.03	3.03
2b	25	33278	29070	0.8736	21.84	4.37	3.16	3.16
3a	50	66556	59500	0.8940	44.70	8.94	5.30	5.30
3b	50	66556	58040	0.8720	43.60	8.72	6.40	6.40
4a	75	99834	88240	0.8839	66.29	13.26	8.71	8.71
4b	75	99834	87910	0.8806	66.04	13.21	8.96	8.96
5a	100	133112	111370	0.8367	83.67	16.73	16.33	16.33
5b	100	133112	112990	0.8488	84.88	16.98	15.12	15.12
6a	125	166390	133380	0.8016	100.20	20.04	24.80	24.80
6b	125	166390	140490	0.8443	105.54	21.11	19.46	19.46
Al, pH 5.5, I 0.05								
1a	0	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0	0
2a	25	38198	30720	0.8042	20.11	4.02	4.89	4.89
2b	25	38198	31040	0.8126	20.32	4.06	4.68	4.68
3a	50	76396	61410	0.8038	40.19	8.04	9.81	9.81
3b	50	76396	63560	0.8320	41.60	8.32	8.40	8.40
4a	75	114594	90880	0.7931	59.48	11.90	15.52	15.52
4b	75	114594	92070	0.8034	60.26	12.05	14.74	14.74
5a	100	152792	117610	0.7697	76.97	15.39	23.03	23.03
5b	100	152792	127010	0.8313	83.13	16.63	16.87	16.87
6a	125	190990	135620	0.7101	88.76	17.75	36.24	36.24
6b	125	190990	138770	0.7266	90.82	18.16	34.18	34.18

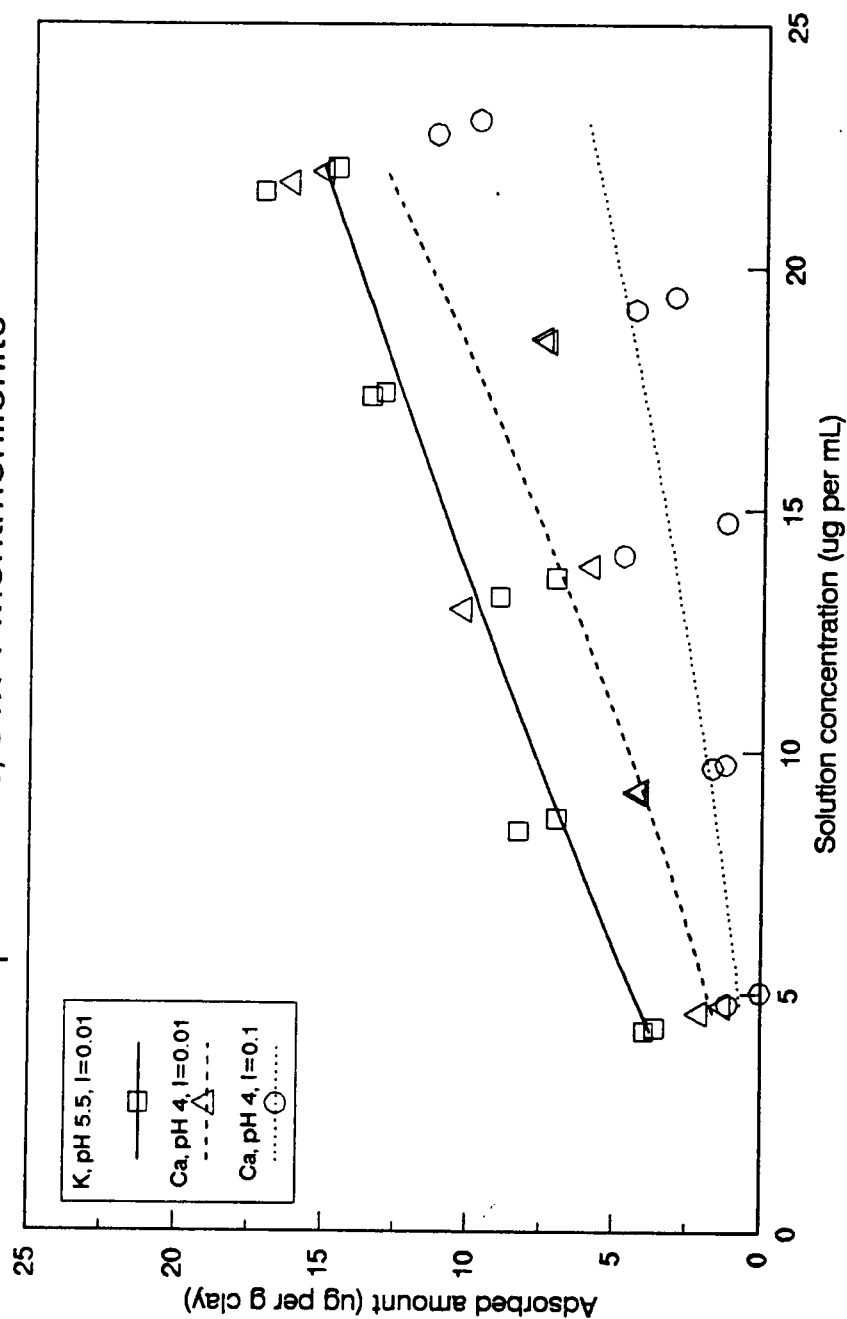
Adsorption Isotherm Naphthalene/SHCa-1 Hectorite



Adsorption Isotherm Data for STx-1 Montmorillonite

Adsorption Isotherm Data for STX-1 Montmorillonite							Naphthalene
Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL		Naphthalene concentration (ug per 5 mL)	Naphthalene concentration (ug per mL)	Naphthalene adsorbed (by difference) (ug per g clay)
			solution after 24 hours	Percent DPM's in solution			
K, pH 5.5, I 0.01	0	0	0	0	0	0	0
1a	0	0	0	0	0	0	0
1b	25	41332	35400	0.8565	21.41	4.28	3.59
2a	25	41332	34840	0.8429	21.07	4.21	3.93
2b	50	82664	68970	0.8343	41.72	8.34	8.28
3a	50	82664	71110	0.8602	43.01	8.60	6.99
3b	75	123996	112340	0.9060	67.95	13.59	7.05
4a	75	123996	109150	0.8803	66.02	13.20	8.98
4b	100	165328	143830	0.8700	87.00	17.40	13.00
5a	100	165328	143060	0.8653	86.53	17.31	13.47
5b	125	206660	178280	0.8627	107.83	21.57	17.17
6a	125	206660	182350	0.8824	110.30	22.06	14.70
6b							
Ca, pH 4, I 0.01							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	41062	39020	0.9503	23.76	4.75	1.24
2b	25	41062	37710	0.9184	22.96	4.59	2.04
3a	50	82124	75380	0.9179	45.89	9.18	4.11
3b	50	82124	75170	0.9153	45.77	9.15	4.23
4a	75	123186	106380	0.8636	64.77	12.95	10.23
4b	75	123186	113570	0.9219	69.15	13.83	5.85
5a	100	164248	151640	0.9232	92.32	18.46	7.68
5b	100	164248	151920	0.9249	92.49	18.50	7.51
6a	125	205310	178640	0.8701	108.76	21.75	16.24
6b	125	205310	180540	0.8794	109.92	21.98	15.08
Ca, pH 4, I 0.01							
1a	0	0	0	0	0	0	0
1b	0	0	0	0	0	0	0
2a	25	38086	36400	0.9557	23.89	4.78	1.11
2b	25	38086	38210	1.0033	25.08	5.02	-0.08
3a	50	76172	74300	0.9754	48.77	9.75	1.23
3b	50	76172	73630	0.9666	48.33	9.67	1.67
4a	75	114258	107020	0.9367	70.25	14.05	4.75
4b	75	114258	112350	0.9833	73.75	14.75	1.25
5a	100	152344	147670	0.9693	96.93	19.39	3.07
5b	100	152344	145610	0.9558	95.58	19.12	4.42
6a	125	190430	173290	0.9100	113.75	22.75	11.25
6b	125	190430	175490	0.9215	115.19	23.04	9.81

Adsorption Isotherm Naphthalene/STx-1 Montmorillonite



Adsorption Isotherm Data for No Clay Blanks

Sample Identification	Naphthalene added (ug)	DPM's added	DPM's in 5 mL.		Naphthalene concentration (ug per 5 mL.)	Naphthalene concentration (ug per mL)	Naphthalene adsorbed (by difference) (ug per g clay)
			solution after 24 hours	Percent DPM's in solution			
K, pH 5.5, I 0.01	0	0	0	0	0	0	0
1a	0	0	0	0	0	0	0
1b	25	41332	41360	1.0007	25.02	5.00	-0.02
2a	25	41332	41490	1.0038	25.10	5.02	-0.10
2b	50	82664	83130	1.0301	51.50	10.30	-1.50
3a	50	82664	78560	0.9504	47.52	9.50	2.48
3b	75	123996	124160	1.0013	75.10	15.02	-0.10
4a	75	123996	125620	1.0131	75.98	15.20	-0.98
4b	100	165328	164470	0.9948	99.48	19.90	0.52
5a	100	165328	160550	0.9711	97.11	19.42	2.89
5b	125	206660	202190	0.9784	122.30	24.46	2.70
6a	125	206660	201010	0.9727	121.58	24.32	3.42
6b							

APPENDIX B

MINERALIZATION ASSAYS: RAW DATA

Naphthalene Mineralization Data for KGa-2 Kaolinite (1g per vial)

Sample Number	CO2 Trap DPM's	Hexane DPM's	Water DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)	
ODa		612	48858	2269	51739	1.25	1.41	0.14	105.76
ODb		828	57911	2228	60967	1.69			124.63
ODc		626	45870	2499	48995	1.28			100.15
ONDa		530	44854	2000	47384	1.08			96.86
ONDb		628	43474	2111	46213	1.28			94.47
1Da	lost	2969	41214	2865	47048	6.07	6.94	0.87	96.17
1Db									
1Dc		3818	41425	2031	47274	7.80			96.64
1NDa		872	46917	1577	49366	1.78			100.91
1NDb		998	47706	2102	50806	2.04			103.86
2Da		3904	41203	2098	47205	7.98	10.10	2.21	96.49
2Db		3816	30266	1739	35821	7.80			73.22
2Dc		7099	47029	2309	56437	14.51			115.37
2NDa		1236	49180	1513	51929	2.53			106.15
2NDb		1201	49329	1895	52425	2.46			107.16
3Da		6931	42665	2269	51865	14.17	16.39	1.79	106.02
3Db		7370	36148	2575	46093	15.07			94.22
3Dc		9756	37853	3162	50771	19.94			103.78
3NDa		1006	51622	1881	54509	2.06			111.42
3NDb		1137	54013	1764	56914	2.32			116.34
4Da		12177	34369	2615	49161	24.89	25.28	1.55	100.49
4Db		13763	34020	2633	50416	28.13			103.06
4Dc		11155	36055	2435	49645	22.80			101.48
4NDa		972	57397	1353	59722	1.99			122.08
4NDb		1169	53512	1606	56287	2.39			115.06
5Da		18601	24361	3086	46048	38.02	43.05	2.61	94.13
5Db		22889	21002	3126	47017	46.79			96.11
5Dc		21697	21753	3263	46713	44.35			95.49
5NDa		1049	46508	1445	49002	2.14			100.17
5NDb		4053	50214	1379	51593	8.28			105.46
6Da		29570	11415	3648	44633	60.45	55.71	3.90	91.24
6Db		28721	13421	3580	45722	58.71			93.46
6Dc		23462	17520	3774	44756	47.96			91.49
6NDa		1136	46971	1527	49634	2.32			101.46
6NDb		936	52005	1616	54557	1.91			111.52

Sample Number Identification Key

D = Degradors (SR)

ND = Non-Degradors (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

Naphthalene Mineralization Data for KGa-2 Kaolinite (1g per vial).....continued

Sample Number	CO2 Trap DPM's	Hexane DPM's	Water DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)
7Da	27716	10579	4472	42767	56.66	59.47	3.22	87.42
7Db	27328	11965	3698	42991	55.86			87.88
7Dc	32240	8306	3921	44467	65.90			90.90
7NDa	1170	47863	1905	50938	2.39			104.13
7NDb	1147	49517	1907	52571	2.34			107.46
8Da	32407	9680	3903	45990	66.24	60.55	3.20	94.01
8Db	26994	9686	4093	40773	55.18			83.35
8Dc	29463	10237	3599	43299	60.23			88.51
8NDa	982	47940	1473	50395	2.01			103.02
8NDb	936	53516	1283	55735	1.91			113.93
9Da	34376	7325	3754	45455	70.27	63.17	4.57	92.92
9Db	26722	11822	3248	41792	54.62			85.43
9Dc	31609	7577	3552	42738	64.61			87.36
9NDa	995	51135	1502	53632	2.03			109.63
9NDb	1027	47898	1921	50846	2.10			103.94
10Da	34178	6221	4048	44447	69.87	70.79	1.86	90.86
10Db	36389	4824	4155	45368	74.38			92.74
10Dc	33328	5809	4058	43195	68.13			88.30
10NDa	1048	45760	2366	49174	2.14			100.52
10NDb	1121	46154	3266	50541	2.29			103.31
11Da	33909	5571	3517	42997	69.32	69.37	2.69	87.89
11Db	36225	4781	3728	44734	74.05			91.44
11Dc	31669	7994	3351	43014	64.74			87.93
11NDa	1247	47715	1422	50384	2.55			102.99
11NDb	3368	45176	1603	50147	6.88			102.51
12Da	33381	4971	3297	41649	68.24	67.08	0.59	85.14
12Db	32627	5021	3300	40948	66.69			83.70
12Dc	32439	4690	3654	40783	66.31			83.37
12NDa	1625	43325	1553	46503	3.32			95.06
12NDb	907	42864	1553	45324	1.85			92.65
CSa	3312	46510	1596	51418	6.77			105.11
CSb	916	50130	1767	52813	1.87			107.96
CSc	1150	50007	1742	52899	2.35			108.13
CEa	885	45282	1637	47804	1.81			97.72
CEb	865	49524	2178	52567	1.77			107.46
CEc	980	46251	1509	48740	2.00			99.63

Sample Number Identification Key

D = Degradors (5R)

ND = Non-Degradors (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

Naphthalene Mineralization Data for KGa-2 Kaolinite (4g per vial)

Sample Number	CO2 Trap DPM's	Hexane DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)	
0Da		809	59037	59846	1.46	1.29	0.10	107.97
0Db		720	60808	61528	1.30			111.01
0Dc		619	55545	56164	1.12			101.33
0NDa		569	55733	56302	1.03			101.58
0NDb		636	57463	58099	1.15			104.82
1Da		2333	53998	56331	4.21	4.29	0.21	101.63
1Db		2594	58042	60636	4.68			109.40
1Dc		2207	56718	58925	3.98			106.31
1NDa		631	66205	66836	1.14			120.58
1NDb		701	65672	66373	1.26			119.75
2Da		2926	49763	52689	5.28	6.66	0.81	95.06
2Db		3659	59310	62969	6.60			113.61
2Dc		4483	55814	60297	8.09			108.78
2NDa		772	63337	64109	1.39			115.66
2NDb		933	64908	65841	1.68			118.79
3Da		7108	50632	57740	12.82	13.29	0.81	104.17
3Db		8237	50133	58370	14.86			105.31
3Dc		6746	49625	56371	12.17			101.70
3NDa		796	63754	64550	1.44			116.46
3NDb		904	64595	65499	1.63			118.17
4Da		9685	47257	56942	17.47	16.30	0.76	102.73
4Db		8248	39301	47549	14.88			85.79
4Dc		9177	42264	51441	16.56			92.81
4NDa		761	52806	53567	1.37			96.64
4NDb		845	52095	52940	1.52			95.51
5Da		11139	39917	51056	20.10	22.56	1.73	92.11
5Db		12023	31873	43896	21.69			79.19
5Dc		14351	35385	49736	25.89			89.73
5NDa		777	55632	56409	1.40			101.77
5NDb		824	63702	64526	1.49			116.41
6Da		10607	46279	56886	19.14	19.11	0.54	102.63
6Db		10067	42273	52340	18.16			94.43
6Dc		11100	42223	53323	20.03			96.20
6NDa		771	53217	53988	1.39			97.40
6NDb		842	53944	54786	1.52			98.84

Sample Number Identification Key

D = Degraders (SR)

ND = Non-Degraders (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

Naphthalene Mineralization Data for KGa-2 Kaolinite (4g per vial)....continued

Sample Number	CO ₂ Trap DPM's	Hexane DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)
7Da	12518	40757	53275	22.58	22.05	1.04	96.12
7Db	13045	38411	51456	23.54			92.83
7Dc	11109	41163	52272	20.04			94.31
7NDa	888	58110	58998	1.60			106.44
7NDb	739	61301	62040	1.33			111.93
8Da	12526	32653	45179	22.60	21.85	1.05	81.51
8Db	10966	38722	49688	19.78			89.64
8Dc	12847	39377	52224	23.18			94.22
8NDa	972	64708	65680	1.75			118.50
8NDb	995	56223	57218	1.80			103.23
9Da	10533	36660	47193	19.00	21.34	1.21	85.14
9Db	12784	36087	48871	23.06			88.17
9Dc	12160	30461	42621	21.94			76.89
9NDa	870	58108	58978	1.57			106.40
9NDb	1015	63718	64733	1.83			116.79
10Da	11658	37248	48906	21.03	21.18	0.87	88.23
10Db	10950	63357	74307	19.76			134.06
10Dc	12607	38770	51377	22.74			92.69
10NDa	889	57345	58234	1.60			105.06
10NDb	935	55373	56308	1.69			101.59
11Da	13621	34362	47983	24.57	24.66	0.94	86.57
11Db	12786	37989	50775	23.07			91.61
11Dc	14592	29397	43989	26.33			79.36
11NDa	792	53459	54251	1.43			97.88
11NDb	878	58021	58899	1.58			106.26
12Da	13476	32708	46184	24.31	23.21	0.64	83.32
12Db	12868	29644	42512	23.22			76.70
12Dc	12250	27566	39816	22.10			71.83
12NDa	811	51748	52559	1.46			94.82
12NDb	849	53463	54312	1.53			97.99
CSa	901	57045	57946	1.63			104.54
CSb	890	54204	55094	1.61			99.40
CSc	827	51780	52607	1.49			94.91
CEa	1067	50032	51099	1.93			92.19
CEb	953	49854	50807	1.72			91.66
CEc	989	46757	47746	1.78			86.14

Sample Number Identification Key

D = Degraders (SR)

ND = Non-Degraders (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

Naphthalene Mineralization Data for SAz-1 Montmorillonite (1g per vial)

Sample Number	CO ₂ Trap DPM's	Hexane DPM's	Water DPM's	Oxidized DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)
0Da	1972	100123	3134	3104	108333	1.39	1.39	0.02	76.25
0Db	2016	95764	3718	2566	104064	1.42			73.24
0Dc	1933	102061	3175	2894	110063	1.36			77.46
0NDa	1612	101344	3598	2394	108948	1.13			76.68
0NDb	2178	97937	2525	2434	105074	1.53			73.95
1Da	9495	90229	3727	3802	107253	6.68	7.25	0.29	75.49
1Db	10867	87663	4070	4084	106684	7.65			75.09
1Dc	10542	95371	4290	4110	114313	7.42			80.46
1NDa	1937	98486	2855	2440	105718	1.36			74.41
1NDb	1962	104908	3027	2396	112293	1.38			79.03
2Da	22442	81463	4224	7948	116077	15.80	15.32	0.44	81.70
2Db	22366	74593	5214	7780	109953	15.74			77.39
2Dc	20509	75882	4301	6190	106882	14.43			75.23
2NDa	1479	109735	2735	2266	116235	1.04			81.81
2NDb	1526	104944	2941	2362	111773	1.07			78.67
3Da	38109	44661	5567	9632	97969	26.82	28.83	1.01	68.95
3Db	42494	50377	5920	3826	102617	29.91			72.22
3Dc	42291	54508	5734	9708	112241	29.77			79.00
3NDa	2522	101069	4267	2494	110352	1.78			77.67
3NDb	2851	112919	2926	2326	121022	2.01			85.18
4Da	61745	23556	6190	14530	106021	43.46	39.36	2.06	74.62
4Db	52423	24585	5321	13698	96027	36.90			67.59
4Dc	53580	29793	5707	14252	103332	37.71			72.73
4NDa	2822	105724	3395	2456	114397	1.99			80.52
4NDb	2711	111515	4788	2848	121862	1.91			85.77
5Da	58573	24010	5898	15260	103741	41.23	49.70	4.39	73.02
5Db	79507	21065	6158	15076	121806	55.96			85.73
5Dc	73757	21675	5987	14450	115869	51.91			81.55
5NDa	2883	111450	3886	2882	121101	2.03			85.23
5NDb	1807	113885	4797	2392	122881	1.27			86.49
6Da	68549	17105	6033	15144	106831	48.25	51.63	2.17	75.19
6Db	72396	17503	6121	16280	112300	50.95			79.04
6Dc	79112	20029	6222	14090	119453	55.68			84.07
6NDa	3122	116587	4072	2570	126351	2.20			88.93
6NDb	3268	109929	3555	2516	119268	2.30			83.94

Sample Number Identification Key

D = Degraders (SR)

ND = Non-Degraders (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

DPM's added to each vial at the start: 142081

Naphthalene Mineralization Data for SAZ-1 Montmorillonite (1g per vial).....continued

Sample Number	CO ₂ Trap DPM's	Hexane DPM's	Water DPM's	Oxidized DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error	Mass Balance per vial (%)
7Da	84454	15185	5727	15148	120514	59.44	60.09	0.81	84.82
7Db	87660	13481	5622	15416	122179	61.70			85.99
7Dc	83996	14061	6498	16860	121415	59.12			85.45
7NDa	3150	116101	4384	2598	126233	2.22			88.85
7NDb	2801	106877	4386	2656	116720	1.97			82.15
8Da	89387	11886	5825	15450	122548	62.91	62.86	0.76	86.25
8Db	87425	11685	6639	15300	121049	61.53			85.20
8Dc	91145	11885	6608	16816	126454	64.15			89.00
8NDa	3118	112261	5146	2796	123321	2.19			86.80
8NDb	2970	121959	4822	2842	132593	2.09			93.32
9Da	87971	12369	5934	15786	122060	61.92	60.78	2.57	85.91
9Db	91725	10466	6034	15994	124219	64.56			87.43
9Dc	79393	9571	6773	14288	110025	55.88			77.44
9NDa	3050	106493	5891	2882	118316	2.15			83.27
9NDb	2942	114636	6407	3244	127229	2.07			89.55
10Da	93414	8158	6272	16432	124276	65.75	64.84	2.73	87.47
10Db	98092	8208	6460	15658	128418	69.04			90.38
10Dc	84851	8663	6550	15630	115694	59.72			81.43
10NDa	3138	113295	6067	2886	125386	2.21			88.25
10NDb	3308	117912	5959	2646	129825	2.33			91.37
11Da	99494	9157	6889	17696	133236	70.03	64.46	3.04	93.77
11Db	90621	8974	6282	15652	121529	63.78			85.54
11Dc	84641	8132	6035	15262	114070	59.57			80.29
11NDa	2642	108978	6709	2834	121163	1.86			85.28
11NDb	2569	119087	5307	2674	129637	1.81			91.24
12Da	94551	6591	8030	15812	124984	66.55	66.90	1.64	87.97
12Db	99303	6704	6260	15830	128097	69.89			90.16
12Dc	91293	6169	6667	14116	118245	64.25			83.22
12NDa	3862	104798	6742	2892	118294	2.72			83.26
12NDb	3217	105654	6718	3176	118765	2.26			83.59
CSa	3681	145435	2765		151881	2.59			106.90
CSb	3900	143618	2986		150504	2.74			105.93
CSc	3552	122585	3355		129492	2.50			91.14
CEa	2567	86853	6863		96283	1.81			67.77
CEb	3412	96762	7581		107755	2.40			75.84
CEc	3009	91678	6966		101653	2.12			71.55

Sample Number Identification Key

D = Degraders (SR)

ND = Non-Degraders (PB2440)

CS = Control Start (No organisms, acidified at Time 0)

CE = Control End (No organisms, acidified at Time 12)

DPM's added to each vial at the start: 142081

Correlation of bioluminescence with mineralization
Naphthalene mineralization by strain HK44

Sample Number	CO2 Trap DPM's	Water DPM's	Total DPM's per vial	Percent Mineralized	Mean	Std Error
0-0g a	920	14070	14990	6.14	5.25	0.89
0-0g-b	527	11550	12077	4.36		
0-0.2g-a	319	11865	12184	2.62	2.73	0.12
0-0.2g-b	363	12369	12732	2.85		
0-2g-a	312	11802	12114	2.58	2.88	0.31
0-2g-b	405	12306	12711	3.19		
1-0g a	5770	17136	22906	25.19	24.26	0.93
1-0g-b	5347	17577	22924	23.32		
1-0.2g-a	2193	13629	15822	13.86	15.46	1.60
1-0.2g-b	2608	12684	15292	17.05		
1-2g-a	1220	12852	14072	8.67	8.83	0.16
1-2g-b	1170	11844	13014	8.99		
2-0g a	8228	17073	25301	32.52	32.96	0.44
2-0g-b	7980	15918	23898	33.39		
2-0.2g-a	5037	10878	15915	31.65	32.43	0.78
2-0.2g-b	5397	10857	16254	33.20		
2-2g-a	3511	13104	16615	21.13	20.35	0.78
2-2g-b	3572	14679	18251	19.57		

APPENDIX C

BIOLUMINESCENCE ASSAYS: RAW DATA

Light response characterization data
Bioluminescence response to salicylate by strain 5R

Full-strength YEPG media

Sample number	Time:	Bioluminescence, nAmps																			
		0	0.33	0.67	1.0	1.33	1.67	2.0	2.33	2.67	3.0	3.33	3.67	4.0	4.33	4.67	5.0	5.33	5.67	6.0	6.33
No clay		0.054	0.380	1.75	4.30	7.94	13.2	17.8	21.8	28.2	34.3	43.8	44.8	49.0	38.2	30.5	31.8	72.0	114	36.2	1.50
0.005g		0.055	0.397	1.80	4.34	8.03	12.6	16.2	19.0	23.2	32.4	39.9	38.3	35.0	27.5	24.4	46.8	112	120	80.0	27.0
0.05g		0.055	0.399	1.68	3.82	7.15	12.4	14.9	19.5	25.2	28.8	31.8	33.3	36.0	25.8	23.2	48.1	106	115	78.0	38.3
0.1g		0.057	0.317	1.36	3.17	6.57	11.3	14.7	19.4	22.8	28.2	34.2	34.3	37.0	27.0	21.0	43.5	102	120	91.0	61.5
0.2g		0.056	0.304	1.30	3.05	5.98	11.0	13.9	18.0	22.8	28.6	35.2	36.6	37.5	28.9	21.7	35.9	80.2	108	103	76.0
0.4g		0.062	0.276	1.19	2.90	5.60	10.3	13.8	18.0	23.2	28.5	33.8	35.7	38.0	32.2	28.0	35.5	76.0	108	107	80.00

One-quarter-strength YEPG media

Sample number	Time:	Bioluminescence, uAmps																			
		0	0.33	0.67	1.0	1.33	1.67	2.0	2.33	2.67	3.0	3.33	3.67	4.0	4.33	4.67	5.0	5.33	5.67	6.0	6.33
No clay	0.003g	0.063	0.193	1.45	4.53	11.8	19.3	25.8	30.2	41.4	48.8	55.0	76.3	97.0	140	203	320	502	672	830	873
0.005g	0.068	0.203	1.35	4.36	13.1	20.0	25.0	31.5	40.4	49.3	64.3	83.0	108	138	192	288	508	733	843	938	
0.05g	0.067	0.166	1.17	4.08	11.1	18.7	23.8	29.8	40.8	50.0	63.8	82.0	99.0	128	212	328	513	642	800	850	
0.1g	0.067	0.175	1.20	4.24	11.5	17.3	21.5	26.8	38.0	46.4	56.4	77.5	88.0	120	205	318	443	572	745	790	
0.2g	0.060	0.191	1.25	4.23	9.70	16.0	18.2	25.9	36.8	45.8	58.1	75.0	93.0	110	188	288	442	568	718	750	
0.4g	0.073	0.164	0.930	3.40	8.15	14.0	16.8	24.2	28.4	37.2	45.2	60.0	76.0	112	173	250	362	433	560	570	

Light response characterization data
Bioluminescence response to naphthalene by strain HK44

Sample number	Time: 0			0.25			0.5			0.75			1.0		
	Mean			Mean			Mean			Mean			Mean		
	Std Error			Std Error			Std Error			Std Error			Std Error		
No clay a	2.6	2.83	0.12	10.3	11.00	0.36	53.0	55.33	1.23	108	112.7	2.33	115	126.7	6.17
No clay b	2.9			11.2			55.8			115			136		
No clay c	3.0			11.5			57.2			115			129		
0.005g a	2.8	2.87	0.07	9.8	10.13	0.24	48.6	49.30	0.51	102	102.7	0.67	131	129.3	1.67
0.005g b	2.8			10.0			50.3			104			126		
0.005g c	3.0			10.6			49.0			102			131		
0.05g a	2.0	2.03	0.03	7.4	7.23	0.12	33.0	33.67	0.93	76.0	71.17	2.46	100	103.3	1.76
0.05g b	2.1			7.0			32.5			68.0			106		
0.05g c	2.0			7.3			35.5			69.5			104		
0.1g a	1.6	1.67	0.03	5.4	5.83	0.26	27.3	28.30	0.51	58.5	60.27	0.91	91.4	96.57	3.52
0.1g b	1.7			5.8			29.0			60.8			95.0		
0.1g c	1.7			6.3			28.6			61.5			103		
0.4g a	1.3	1.40	0.06	5.4	5.43	0.09	23.2	22.83	0.52	42.8	45.77	1.65	85.0	81.73	2.07
0.4g b	1.4			5.3			21.8			46.0			77.9		
0.4g c	1.5			5.6			23.5			48.5			82.3		
1.0g a	0.87	0.87	0.04	2.9	3.00	0.06	10.7	11.73	0.89	22.9	24.17	1.58	42.9	46.40	2.05
1.0g b	0.80			3.0			11.0			27.3			50.0		
1.0g c	0.95			3.1			13.5			22.3			46.3		

Bioluminescence response to naphthalene by strain HK44 in the absence of clay

Sample number	Time: 0			0.25			0.5			0.75			1.0		
	Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No naphth a	1.7	1.80	0.06	3.6	3.47	0.14	4.5	4.37	0.09	5	5.0	0.15	7	6.4	0.15
No naphth b	1.8			3.6			4.4			5			6		
No naphth c	1.9			3.2			4.2			5			6		
6 mg/L a	4.5	4.53	0.03	19.4	19.03	0.42	90.5	89.77	2.00	205	198.3	4.81	402	390.3	5.84
6 mg/L b	4.5			18.2			86.0			189			384		
6 mg/L c	4.6			19.5			92.8			201			385		
3 mg/L a	4.3	4.02	0.31	19.0	19.13	0.19	75.5	75.27	1.48	135.0	138.00	1.73	153	153.7	3.48
3 mg/L b	3.4			18.9			72.6			138.0			148		
3 mg/L c	4.4			19.5			77.7			141.0			160		
1.5 mg/L a	3.6	3.62	0.03	15.3	15.13	0.33	47.9	46.37	0.78	55.0	53.67	0.88	113.0	116.67	3.67
1.5 mg/L b	3.6			14.5			45.8			52.0			113.0		
1.5 mg/L c	3.7			15.6			45.4			54.0			124		
0.75 mg/L a	2.4	2.30	0.08	9.0	8.57	0.34	24.3	23.80	0.29	35.0	34.00	0.58	43.0	42.67	0.33
0.75 mg/L b	2.2			7.9			23.3			33.0			43.0		
0.75 mg/L c	2.4			8.8			23.8			34.0			42.0		

Correlation of bioluminescence with mineralization
Bioluminescence response to naphthalene by strain HK44

Sample number	Time:	Bioluminescence, nAmps					
		0	0.25	0.5	0.75	1.0	
No clay a		0.23	0.30	0.04	1.00	1.10	0.05
No clay b		0.33	1.13		1.80	2.01	0.10
No clay c		0.33	1.16		2.10	2.12	3.15
0.2g a		0.23	0.23	0.04	0.78	0.90	0.06
0.2g b		0.17	0.92		1.60	1.52	0.12
0.2g c		0.31	1.00		1.68		2.10
2.0g a		0.10	0.17	0.04	0.22	0.25	0.02
2.0g b		0.18	0.23		0.40	0.42	0.07
2.0g c		0.24	0.29		0.55	0.76	0.17
					1.08		0.34
							1.05
							1.90
							3.25
							4.30
							5.25
							4.3
							0.58
							6.1
							0.39
							6.68
							6.15

Sample number	Time:	Bioluminescence, nAmps					
		1.25	1.5	1.75	2		
No clay a		12.20	11.7	0.36	18.8	19.3	0.37
No clay b		11.00	20.0		21.7	22.6	0.58
No clay c		11.90	19.0		23.7	21.3	20.2
0.2g a		6.10	6.8	0.44	9.85	11.1	0.89
0.2g b		6.55	10.6		15.3	17.4	1.50
0.2g c		7.60	12.8		16.6	25.2	30.9
2.0g a		1.25	2.4	0.57	1.90	3.8	0.97
2.0g b		2.92	4.85		2.73	5.8	1.55
2.0g c		3.00	4.75		7.60	11.5	10.9
					7.10		8.8
							2.46
							2.40
							20.1
							0.75
							18.7
							20.2
							26.3
							3.85
							11.5
							10.9

Test for change in cell numbers
Bioluminescence response to naphthalene by strain HK44

Sample number	Time	Bioluminescence, nAmps														
		0			0.33			0.67			1.00			1.33		
		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No clay a		0.06	0.06	0.00	0.09	0.09	0.00	0.13	0.13	0.00	0.22	0.2	0.01	0.35	0.3	0.02
No clay b		0.06			0.09			0.13			0.19			0.30		
No clay c		0.06			0.09			0.14			0.20			0.34		
0.005g a		0.06	0.06	0.00	0.09	0.09	0.00	0.13	0.13	0.00	0.19	0.2	0.00	0.33	0.3	0.01
0.005g b		0.06			0.09			0.13			0.19			0.32		
0.005g c		0.06			0.10			0.14			0.20			0.34		
0.05g a		0.06	0.06	0.00	0.09	0.09	0.00	0.13	0.12	0.01	0.21	0.19	0.01	0.30	0.3	0.01
0.05g b		0.06			0.09			0.11			0.17			0.27		
0.05g c		0.06			0.10			0.13			0.18			0.30		
0.1g a		0.06	0.06	0.00	0.09	0.09	0.00	0.11	0.12	0.01	0.16	0.17	0.01	0.27	0.28	0.01
0.1g b		0.06			0.09			0.13			0.18			0.29		
0.1g c		0.06			0.09			0.12			0.18			0.28		
0.2g a		0.06	0.06	0.00	0.07	0.08	0.00	0.12	0.11	0.01	0.17	0.17	0.00	0.28	0.26	0.02
0.2g b		0.06			0.08			0.10			0.17			0.23		
0.2g c		0.06			0.08			0.12			0.18			0.27		
0.4g a		0.06	0.06	0.00	0.07	0.08	0.00	0.12	0.12	0.00	0.16	0.17	0.01	0.26	0.27	0.01
0.4g b		0.06			0.08			0.12			0.17			0.26		
0.4g c		0.06			0.08			0.12			0.18			0.29		

Sample number	Time	1.67			2.00			2.33			2.67			3.00		
		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No clay a		0.55	0.5	0.01	0.85	0.8	0.02	1.10	1.1	0.02	1.50	1.5	0.03	2.00	1.9	0.06
No clay b		0.51			0.79			1.05			1.41			1.80		
No clay c		0.55			0.83			1.09			1.45			1.97		
0.005g a		0.53	0.5	0.01	0.78	0.8	0.01	1.02	1.0	0.01	1.44	1.4	0.02	1.83	1.8	0.02
0.005g b		0.51			0.80			1.04			1.43			1.77		
0.005g c		0.52			0.75			1.01			1.38			1.78		
0.05g a		0.47	0.5	0.02	0.69	0.7	0.03	0.88	0.9	0.05	1.08	1.1	0.05	1.34	1.4	0.06
0.05g b		0.42			0.60			0.80			1.08			1.43		
0.05g c		0.47			0.69			0.97			1.24			1.55		
0.1g a		0.41	0.44	0.01	0.60	0.62	0.02	0.72	0.78	0.03	0.95	1.02	0.04	1.19	1.15	0.02
0.1g b		0.45			0.65			0.80			1.10			1.12		
0.1g c		0.45			0.61			0.83			1.01			1.14		
0.2g a		0.40	0.41	0.02	0.58	0.57	0.01	0.70	0.71	0.02	0.91	0.89	0.03	1.09	1.10	0.02
0.2g b		0.38			0.55			0.67			0.84			1.07		
0.2g c		0.44			0.58			0.75			0.92			1.13		
0.4g a		0.39	0.40	0.01	0.59	0.56	0.02	0.70	0.67	0.03	0.84	0.85	0.01	1.00	1.13	0.09
0.4g b		0.39			0.52			0.61			0.84			1.29		
0.4g c		0.43			0.56			0.70			0.88			1.09		

Test for change in cell numbers.....continued
 Bioluminescence response to naphthalene by strain HK44

Bioluminescence, nAmps

Sample number	Time	3.67			4.00			4.33			5.33			5.83		
		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No clay a		3.62	3.6	0.16	5.30	5.0	0.27	6.55	6.2	0.30	11.8	11.0	0.63	15.8	14.5	0.93
No clay b		3.25			4.42			5.60			9.78			12.7		
No clay c		3.79			5.14			6.45			11.5			15.0		
0.005g a		3.29	3.3	0.04	4.75	4.6	0.06	5.98	5.9	0.09	10.5	10.4	0.09	14.5	14.0	0.24
0.005g b		3.20			4.62			6.05			10.4			13.9		
0.005g c		3.33			4.33			5.75			10.2			13.7		
0.05g a		2.15	2.4	0.18	2.75	3.0	0.22	3.40	3.8	0.33	5.35	6.2	0.65	6.72	7.6	0.83
0.05g b		2.23			2.84			3.46			5.66			6.80		
0.05g c		2.74			3.44			4.42			7.45			9.24		
0.1g a		1.80	1.85	0.06	2.12	2.18	0.08	2.78	2.66	0.07	3.95	3.92	0.06	4.60	4.52	0.06
0.1g b		1.77			2.08			2.55			3.80			4.40		
0.1g c		1.97			2.34			2.65			4.00			4.55		
0.2g a		1.65	1.72	0.04	2.03	2.01	0.03	2.32	2.36	0.04	3.28	3.32	0.06	3.50	3.67	0.09
0.2g b		1.72			1.96			2.45			3.44			3.75		
0.2g c		1.78			2.05			2.32			3.25			3.77		
0.4g a		1.55	1.81	0.13	1.88	2.05	0.09	2.14	2.30	0.09	2.95	3.23	0.14	3.14	3.41	0.14
0.4g b		2.00			2.18			2.45			3.39			3.60		
0.4g c		1.88			2.10			2.32			3.35			3.50		

Sample number	Time	6.33			6.83			7.33		
		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No clay a		21.0	19.1	1.42	30.0	30.0	0.00	45.5	45.5	0.00
No clay b		16.3								
No clay c		19.9								
0.005g a		19.5	18.6	0.49	27.8	26.0	0.96	42.5	39.6	1.46
0.005g b		18.5			25.8			38.5		
0.005g c		17.8			24.5			37.8		
0.05g a		8.00	8.7	0.84	9.07	9.07	0.00	12.4	12.4	0.00
0.05g b		7.75								
0.05g c		10.4								
0.1g a		5.00	4.94	0.12	5.85	5.85	0.00	7.35	7.35	0.00
0.1g b		4.71								
0.1g c		5.10								
0.2g a		4.30	4.17	0.07	4.83	4.75	0.08	6.00	5.67	0.18
0.2g b		4.07			4.82			5.40		
0.2g c		4.15			4.60			5.60		
0.4g a		3.45	3.75	0.15	4.25	4.25	0.00	4.45	4.45	0.00
0.4g b		3.85								
0.4g c		3.96								

Test for change in cell numbers
Plate counts

Sample number	Time:	Plate Counts					
		0		2.00		6.33	
		Mean		Mean		Mean	
No clay a		3.00E+08	3.30E+08	5.90E+08	5.50E+08	5.90E+08	5.65E+08
No clay b		3.60E+08		5.10E+08		5.40E+08	
Suspension							
0.05g a		4.30E+08	4.20E+08	5.20E+08	5.10E+08	6.60E+08	6.15E+08
0.05g b		4.10E+08		5.00E+08		5.70E+08	
0.1g a		4.80E+08	4.05E+08	4.80E+08	5.35E+08	7.40E+08	6.60E+08
0.1g b		3.30E+08		5.90E+08		5.80E+08	
0.4g a		4.30E+08	4.10E+08	5.00E+08	5.25E+08	8.80E+08	6.35E+08
0.4g b		3.90E+08		5.50E+08		3.90E+08	
Supernatant							
0.05g a		3.90E+08	3.80E+08	5.60E+08	4.90E+08	7.30E+08	7.55E+08
0.05g b		3.70E+08		4.20E+08		7.80E+08	
0.1g a		3.60E+08	3.35E+08	4.40E+08	4.60E+08	6.40E+08	6.40E+08
0.1g b		3.10E+08		4.80E+08			
0.4g a		4.40E+08	4.55E+08	5.20E+08	4.70E+08	7.00E+08	5.85E+08
0.4g b		4.70E+08		4.20E+08		4.70E+08	

Test for change in cell numbers

Optical densities and corresponding bioluminescence readings

Sample number	Optical Density at 546 nm	Bioluminescence Response, nAmps
No clay a	0.24	1110
No clay b	0.26	520
No clay c	0.23	660
No clay d	0.21	340
0.1g a	0.31	460
0.1g b	0.29	320
0.1g c	0.28	330
0.1g d	0.27	175
0.4g a	0.27	270
0.4g b	0.27	230
0.4g c	0.24	170
0.4g d	0.22	140
1.0g a	0.24	101
1.0g b	0.26	41
1.0g c	0.20	46
1.0g d	0.21	86

Test for external mass transfer limitations
 Bioluminescence response to naphthalene by strain HK44

	Time:	Bioluminescence, nAmps					
		0.00	0.25	0.50	0.75	1.00	1.25
Vials at rest							
No clay		0.65	1.82	3.41	5.40	9.10	19.80
0.1g		0.55	1.43	2.80	4.70	7.20	14.50
0.4g		0.48	1.23	2.45	4.20	6.90	11.60
1.0g		0.32	0.68	1.58	2.90	4.60	8.00
Vials shaken at 100 rpm							
No clay		0.22	1.62	5.02	10.30	18.90	40.50
0.1g		0.20	1.00	2.90	6.10	10.70	19.20
0.4g		0.17	0.66	2.20	3.80	7.50	14.20
1.0g		0.10	0.25	1.15	1.80	5.20	8.50
Vials vortexed							
		0.41	1.91	4.80	8.90	17.80	44.50
0.1g		0.30	1.02	2.20	4.30	7.60	22.50
0.4g		0.17	0.63	1.29	2.80	4.50	13.60
1.0g		0.15	0.22	0.22	0.32	0.48	0.85

Test for intraparticle diffusion

Bioluminescence response to naphthalene by strain HK44

		Bioluminescence, nAmps					
Time:		0.00	0.25	0.50	0.75	1.00	1.25
Naphthalene applied 24 hours before assay							
No clay		0.22	1.62	5.02	10.30	18.90	40.50
0.1g		0.20	1.00	2.90	6.10	10.70	19.20
0.4g		0.17	0.66	2.20	3.80	7.50	14.20
1.0g		0.10	0.25	1.15	1.80	5.20	8.50
Naphthalene applied immediately before assay							
No clay		0.31	1.44	4.55	10.70	20.70	43.50
0.1g		0.25	0.85	2.55	6.00	11.00	20.20
0.4g		0.20	0.65	1.73	3.90	8.80	15.30
1.0g		0.12	0.30	0.75	1.80	5.00	9.30

Change in clay:microorganism ratio
Bioluminescence response to naphthalene by strain HK44

1.00E+10 cells added per vial

Sample number	Time:	0	0.33	0.67	1.0	1.33	1.67	2.0	2.33	2.67	3.0	3.33	3.67	4.0	4.33	4.67	5.0
Bioluminescence, nAmps																	
No clay		0.170	0.245	0.320	0.672	2.05	6.55	16.1	29.4	43.4	89.1	36.3	19.2	10.6	7.30	4.07	2.45
0.005g		0.126	0.173	0.215	0.324	0.708	1.59	4.23	8.23	10.0	20.0	30.0	49.1	66.5	81.4	118	15.5
0.05g		0.116	0.169	0.195	0.258	0.580	1.20	2.88	5.72	10.0	12.6	17.7	26.0	36.7	49.5	80.0	68.2
0.1g		0.117	0.148	0.168	0.233	0.528	1.04	2.55	4.92	9.50	10.8	15.5	21.8	28.9	44.0	62.5	87.5
0.2g		0.123	0.148	0.158	0.236	0.528	1.12	2.50	4.60	8.30	10.5	15.2	21.8	27.7	39.0	54.0	78.5
0.4g		0.103	0.146	0.159	0.261	0.550	1.08	2.35	4.33	8.30	10.8	14.1	22.4	25.9	37.4	56.7	77.5

4.00E+08 cells added per vial

Sample number	Time:	0	0.33	0.67	1.0	1.33	1.67	2.0	2.33	2.67	3.0	3.33	3.67	4.0	4.33	4.67	5.0
Bioluminescence, nAmps																	
No clay		0.075	0.127	0.403	1.04	1.97	3.23	4.60	6.06	8.36	10.1	13.5	18.5	24.7	32.0	39.6	48.2
0.005g		0.069	0.118	0.290	0.633	0.980	1.56	2.37	3.42	4.75	6.30	7.75	9.50	10.8	10.5	12.1	14.1
0.05g		0.067	0.114	0.256	0.502	0.947	1.36	2.07	3.22	4.11	5.16	6.38	6.88	7.98	8.72	8.93	9.03
0.1g		0.079	0.119	0.262	0.588	1.13	1.45	2.02	2.65	4.06	4.91	6.29	7.65	8.48	9.63	10.3	10.2
0.2g		0.079	0.108	0.241	0.543	0.885	1.18	1.63	2.38	3.30	4.88	5.47	6.38	7.53	9.25	9.45	10.0
0.4g		0.072	0.117	0.250	0.471	0.787	0.980	1.23	1.91	2.87	4.23	4.67	5.48	6.44	7.96	8.06	7.88

Addition of agents to change clay aggregate stability
 Bioluminescence response to naphthalene by strain HK44

		B i o l u m i n e s c e n c e, n A m p s					
Time:		0.00	0.25	0.50	0.75	1.00	1.25
No additive							
No clay		0.54	1.70	4.50	11.1	22.6	49.2
1.0g		0.13	0.33	1.00	2.50	3.90	5.3
De-stabilizer							
No clay		0.65	2.00	7.80	16.4	24.8	34.0
1.0g		0.16	0.40	1.10	3.00	4.80	6.80
Stabilizer							
No clay		0.25	0.55	1.80	4.40	9.50	21.0
1.0g		0.17	0.35	0.95	1.85	3.00	4.20

Addition of a stabilizing agent at different time points
 Bioluminescence response to naphthalene by strain HK44

		Bioluminescence, nAmps						
Time:		0.00	0.25	0.50	0.75	1.00	1.25	1.50
No stabilizer								
No clay		0.37	1.25	3.05	6.22	11.8	25.2	53.0
0.1g		0.31	0.86	2.18	4.04	7.25	12.0	19.1
0.4g		0.28	0.79	1.49	3.44	6.90	11.2	16.9
1.0g		0.20	0.48	1.09	2.05	3.55	6.20	10.0
Stabilizer added at 0 hours								
No clay		0.38	1.13	2.75	5.00	8.83	17.0	34.2
0.1g		0.32	0.87	2.25	4.15	5.10	12.5	23.8
0.4g		0.33	0.75	1.67	2.45	6.45	12.2	20.8
1.0g		0.24	0.40	0.92	1.40	3.00	7.30	11.2
Stabilizer added at 0.75 hours								
No clay		0.43	1.42	3.50	7.04	10.4	18.0	32.3
0.1g		0.33	1.04	2.48	5.02	7.85	11.8	18.5
0.4g		0.31	0.75	1.61	3.68	7.20	10.3	15.0
1.0g		0.21	0.53	0.12	2.15	5.55	8.40	11.6

Test for particle size effects

Bioluminescence response to naphthalene by strain HK44

		Bioluminescence, nAmps						
	Time:	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Vials at rest								
No sand		0.55	1.62	3.30	5.30	8.70	18.0	43.3
0.1g		0.58	1.62	3.20	5.20	8.90	17.0	50.0
0.4g		0.54	1.48	2.70	4.50	7.70	16.2	37.0
1.0g		0.54	1.40	2.60	4.30	6.90	14.3	37.0
Vials shaken at 100 rpm								
No sand		0.90	1.70	2.94	4.50	7.70	15.7	36.0
0.1g		0.75	1.60	2.80	4.70	7.80	15.8	36.0
0.4g		0.70	1.52	2.60	4.10	6.90	13.9	31.0
1.0g		0.68	1.48	2.50	4.10	6.90	14.0	31.0
Vials vortexed								
No sand		0.80	1.57	2.60	4.00	6.40	12.9	31.0
0.1g		0.80	1.58	2.60	3.70	5.70	11.8	30.0
0.4g		0.77	1.44	2.29	3.10	4.90	9.60	25.0
1.0g		0.73	1.20	1.85	2.50	3.80	7.60	19.0

Use of immobilized cells and different mixing conditions
 Bioluminescence response to naphthalene by strain HK44

		Bioluminescence, n A m p s					
Time:		0.00	0.25	0.50	0.75	1.00	1.25
Vials at rest							
No clay		0.06	0.06	0.24	0.58	1.05	1.70
0.1g		0.07	0.07	0.30	0.66	1.20	1.30
0.4g		0.08	0.07	0.44	1.36	1.50	1.80
1.0g		0.06	0.10	0.50	1.35	3.40	3.30
Vials shaken at 100 rpm							
No clay		0.05	0.06	0.15	1.22	3.75	5.10
0.1g		0.10	0.07	0.40	2.20	5.15	5.70
0.4g		0.10	0.08	0.51	2.75	5.50	4.70
1.0g		0.06	0.07	0.48	2.42	4.70	3.90

Use of immobilized cells (second test)
Bioluminescence response to naphthalene by strain HK44

Sample number	Time: 0			0.25			0.5			0.75			1.0			1.25		
	Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error		Mean	Std Error	
No clay a	0.89	1.23	0.18	1.3	1.50	0.12	1.9	2.37	0.26	3.8	4.4	0.33	9.1	9.3	0.19	20.3	22.3	1.71
No clay b	1.3			1.7			2.8			4.6			9.7			20.9		
No clay c	1.5			1.5			2.4			4.9			9.2			25.7		
0.1g a	0.88	1.23	0.17	1.2	1.53	0.20	1.8	2.60	0.53	3.8	5.3	1.09	8.3	11.0	1.91	19.9	25.4	3.33
0.1g b	1.4			1.9			3.6			7.4			14.7			31.4		
0.1g c	1.4			1.5			2.4			4.6			10.0			25.0		
0.4g a	1.2	1.37	0.09	1.7	1.83	0.07	3.5	3.57	0.03	8.1	7.67	0.38	21.4	17.90	1.94	31.8	33.47	0.85
0.4g b	1.5			1.9			3.6			8.0			14.7			34.0		
0.4g c	1.4			1.9			3.6			6.9			17.6			34.6		
1.0g a	1.1	1.33	0.15	1.4	1.93	0.32	2.6	3.73	0.70	6.8	8.20	0.98	16.1	18.33	1.51	29.9	39.63	4.90
1.0g b	1.6			2.5			5.0			10.1			21.2			45.5		
1.0g c	1.3			1.9			3.6			7.7			17.7			43.5		

VITA

Rebecca Mirsky Pullen was born in San Rafael, California on August 23, 1956. She spent her first 20 years in the San Francisco Bay Area, interrupted by two years in Invercargill, New Zealand, six months in Montreal, and two years in Rockville, Maryland. From an early age, her career aspirations have leaned toward animals and the environment, including ornithology, marine biology and veterinary medicine.

In September, 1973, she entered Dominican College of San Rafael, six months before receiving her high school diploma from Redwood High School. In October, 1976, she transferred to the University of California at Davis to complete a Bachelor of Science degree with an individualized major of Animal Behavior, and to apply to veterinary school. After four years of rejected vet school applications, supported by stints as a Kelly Girl, she landed a job as a piping and mechanical systems estimator at an engineering firm in Sacramento, California. Soon after, relinquishing all hope of becoming a veterinarian, she accepted a position as a cost engineer with the Bechtel Corporation, and moved back to San Francisco. Her assignments with Bechtel took her to Richland, Washington, where she met her husband, Jeff, and later to Vandenberg Air Force Base, California; Kennedy Space Center, Florida; and Calvert City, Kentucky, where she spent two years on her first environmental project. At the end of that project, she was transferred to Oak Ridge, Tennessee, where she resigned to become a full-time student at the University of Tennessee in Knoxville.

After completing a Master of Science degree in environmental engineering, she applied for and received a three year doctoral fellowship from the U.S. Department of Agriculture. While completing the work for a Doctorate in civil engineering, she also received a second Bachelor of Science degree in engineering science and mechanics.

Now, with more degrees than she knows what to do with, she is ready to re-enter the job market, and secure a vastly lucrative position which would allow her long-suffering husband to retire in style.