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By

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ABSTRACT

BIOSTABILITY AND BIOAVAILABILITY OF SOIL- AND CLAY-EXCHANGED HEXADECYLTRIMETHYLAMMONIUM

By

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Soil modification with quaternary ammonium compounds such as hexadecyltrimethylammonium (HDTMA) enhances non-ionic contaminant (NOC) sorption. Coupling NOC immobilization with *in situ* bioremediation of the NOCs would constitute a comprehensive soil restoration technology. The biostability of soil- and clay-bound HDTMA was evaluated in a variety of soils and in pure cultures of HDTMA-degrading microorganisms. Studies indicated that HDTMA-complexes were generally biologically stable. The extent of mineralization was dependent on the nature of the exchange sites (organo-clay complexes and especially those with internal exchange sites are more stable than free HDTMA) and on environmental conditions (subsurface soils offer more stability). A mineralization kinetics approach assessed sorbed HDTMA bioavailability by comparing sorbed and solution phase HDTMA mineralization rates. Based on this model, bound HDTMA was available while availability was minimized by HDTMA exchange in dilute salt solutions and by increasing the cation exchange capacity:HDTMA to greater than 1:1.

TO MOM AND DAD

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LITERATURE REVIEW

The soil organic matter (SOM) fraction of soils plays a predominant role in the sorption of non-ionic organic contaminants (NOCs) from water by soil as non-polar interactions between solute molecules and the SOM result in NOC solubilization and partitioning into amorphous SOM (8, 9, 23, 25). Sorption depends largely on the solute's water solubility (8, 9) and the SOM content (6, 14, 20, 23). When sorption coefficients for NOCs are normalized on the basis of SOM content (K_{om}), their values remain fairly constant among various soils, reflecting the importance of the SOM in the sorption process (27). Clays have a net negative electrical charge which is balanced by the presence of the strongly hydrated exchangeable cations such as Na^{+}, K^{+}, Mg^{++}, and Ca^{++}. Soil minerals (i.e., clays) are generally ineffective as sorbents of NOCs due to the polar nature of Si-O groups and the presence of hydrated exchange cations (10). Water is so strongly held by the mineral surfaces that NOCs are unable to compete for exchange sites. Therefore, low organic matter surface soils and subsoils have a limited capability to sorb NOCs, increasing the potential for NOC transport.

Recently, Lee et al. (27) developed a soil modification approach for reducing contaminant transport in soils, subsoils, and aquifer materials. Their studies have shown that the sorptive capacity of

soils and subsoils can be greatly increased through the addition of large hydrophobic cationic moieties of the general form $[(CH_3)_3NR]^+$ or $[(CH_3)_2NRR']^+$, where R and R' are large alkyl hydrocarbons, as for example, hexadecyltrimethylammonium (HDTMA), which increase the organic matter content of the soils. Once added to soil, the quaternary ammonium compounds (QUATs), such as HDTMA, displace native inorganic cations from the cation exchange sites of soil clays resulting in the formation of an organic partition phase comprised of the alkyl hydrocarbon groups of the organic cation (e.g., the C-16 groups of HDTMA). By permanently separating the aluminosilicate sheets of the clay minerals, these amendments enhance the uptake of NOCs from soil solution.

Subsurface (B horizon) soils generally contain low concentrations of natural SOM and, in turn, lack the greater sorptive properties associated with surface (A horizon) soils. HDTMA was exchanged onto Marlette soil from both the A and B_t horizons and the sorptive properties of the treated and untreated materials were compared (27); the uptake of benzene, toluene, and ethylbenzene by the treated horizons was significantly enhanced. The relative increase in the sorptive capacity for the treated A horizon was lower than for the B_t horizon due to the higher concentration of natural SOM present in the surface soil. The sorption coefficient (K) increased 15 times, while the organic matter content of the A horizon soil was only approximately doubled through the addition of HDTMA. This suggests that synthetic organic matter (e.g., HDTMA) is more effective than natural SOM in attenuating organic contaminant mobility. K_{om} values, which are used to indicate

the effectiveness of various organic phases on a unit weight basis, also show the increased effectiveness of synthetic organic matter. A comparison of K_{om} values for benzene, toluene, TCE, PCE, and ethylbenzene in untreated A horizon soil (all natural SOM) and the treated B_t horizon (all synthetic organic matter) reveals an increase of 10-30 times for the B_t horizon's sorption coefficients. These data indicate that the organic sorptive phase formed in HDTMA-treated soils is 10 to 30 times more effective on a unit mass basis than natural SOM in removing NOCs from solution. HDTMA forms a low polarity organic phase derived from the interacting C-16 hydrocarbon tails whereas SOM contains more polar groups which lower its effectiveness as a partition phase for NOCs.

Increases in organic matter content, and therefore increases in the sorptive capabilities of organo-clays, are directly proportional to the chain length of the QUAT's alkyl group (27). Large organic cations, such as HDTMA and dioctadecyldimethylammonium (DODMA; R, R' = C_{18}), are more effective than the smaller moieties (e.g., Tetramethylammonium (TMA)) in removing NOCs, such as pentachlorophenol, from solution (5). A partitioning process governs sorption by the more hydrophobic clays (those exchanged with HDTMA or DODMA) in which the cations form an organic phase analogous to an organic solvent phase. Such a phase enhances NOC uptake and hence lowers contaminant levels in the soil solution. Organo-clays modified with the smaller cations, on the other hand, behave as mineral sorbents (5); with decreasing chain length, the exchanged hydrocarbon cations become more isolated on the mineral surface and the partitioning phase becomes less efficient (27).

Jaynes and Boyd (22) related mineralogy to sorbent efficiency by comparing the sorption of aromatic hydrocarbons by different clay minerals exchanged with HDTMA. HDTMA-smectites, -illites, and -vermiculites are all highly effective in the sorption of organic contaminants. Log K_{om} values indicate that HDTMA-clays with the largest basal spacings are the most effective sorbents of substituted benzene rings, naphthalene, and biphenyl. Paraffin and pseudotrimolecular complexes, such as HDTMA-vermiculites and high charge HDTMA-smectites, are distinctly more effective than those HDTMA-clays forming bilayers (e.g., low-charge HDTMA-smectites). Unexpectedly, HDTMA-exchanged illite was found to be nearly as effective as expanding clays with similar CECs. Apparently, externally-bound HDTMA forms a partitioning phase analogous to that formed in the inner layers of expanding clays, thereby accounting for its high sorptive capacity.

Remediation of a polluted site could be accomplished by coupling this immobilization technology with *in situ* bioremediation of the NOCs. *In situ* formation of organo-clay complexes from naturally-occurring clays (i.e., those present in subsoils or aquifer materials) could be achieved by injection of HDTMA into the subsurface to create a sorptive zone that will retard NOC migration and concentrate the target contaminants within a confined area (3). Burris and Antworth (7) have demonstrated the feasibility of this concept by injection of HDTMA into a confined aquifer material; the resultant sorptive barrier effectively immobilized naphthalene in a simulated contaminant plume. Subsequent biodegradation of the immobilized contaminants would constitute a comprehensive soil restoration technology. The presence of indigenous

soil microbes capable of utilizing the NOCs as carbon and energy sources requires maintaining favorable growth conditions in the soil. Often at newly contaminated sites, however, the appropriate species may not be present. An alternative approach is the addition of bacterial degraders possessing the desired physiological characteristics as well as optimization of soil conditions to support bacterial survival and growth (17).

Organo-clays may also be employed as an additional containment (sorptive) zone around hazardous waste containment facilities or chemical storage tanks which currently use bentonite liners as physical barriers to reduce hydraulic conductivity or as barriers around underground storage tanks to protect groundwater supplies (4). In such instances, when inadvertently released solvents or fuels make contact with the modified clay layer, sorption and immobilization will retard further contaminant migration and ultimately reduce the extent of contamination.

QUATs with alkyl chain lengths ranging from C_{12} to C_{18} are commercially valuable chemicals used in fabric softeners, disinfectants, drilling muds, hair care products, and detergents (38). As a result, these compounds are eventually released into wastewater treatment systems and ultimately reach a variety of associated environments including rivers and lakes. They are also highly toxic to bacteria, algae, fish, shrimp, and other aquatic organisms at sub-mM concentrations (2). Tubbing et al. (36) studied the effects of ditallowdimethylammonium chloride (DTDMAC) on indigenous bacteria and phytoplankton in the Rhine River. Bacterial thymidine incorporation, an

indicator of metabolic activity and growth, decreased at DTDMAC concentrations of 0.03 to 0.3 mg L^{-1}, while photosynthesis was adversely affected at concentrations exceeding 0.1 mg L^{-1}. Complete inhibition of both processes occurred at around 10 mg L^{-1} DTDMAC.

The biochemical mechanisms by which cationic surfactants exert their germicidal action have been the focus of much research. However, no complete theory has been developed and it is believed that there may be a variety of actual mechanisms by which surface-active agents attack the cell and that the route may be dependent on both the organism and on the surfactant concentration. Generally, the cause of the toxicity has been attributed to the physical properties of the cations which interact with bacterial cells by binding to lipids (1, 19, 21) or membrane proteins (24). The lipophilicity of the surfactant enhances its ability to traverse the cell wall and exert its toxicity through cell lysis. N-alkyl chain length determines the overall toxicity of these agents to bacteria (15) with antimicrobial activity maximized for substituent chain lengths of 14 to 16 carbon atoms (12). Gilbert et al. (15) proposed that this increased activity is a result of 1) dimerization of long-chain QUATs which enhances cell membrane permeation or 2) dual binding sites on the cell surface for the larger moieties.

Although cationic surfactants have been extensively used as germicidal agents, their widespread use and discharge have been met with the microbial adaptation to (30, 38) and the mineralization of (11, 13, 26, 32, 35, 37) surfactants by indigenous microbes in aquatic and terrestrial habitats. Adaptation to these compounds plays an important role in initiation of degradative capabilities. Ventullo and Larson

(38) showed that initial rates of glucose mineralization in soil samples were reduced after exposure to dodecyltrimethylammonium chloride (DTMAC; R = C_{12}). However, heterotrophic activity eventually recovered and met or exceeded control (no treatment) levels, indicating that one or more tolerant species are selected for initially, followed by a population rebound. In surfactant mineralization studies, an initial exposure to DTMAC exhibited a 24-hour lag period prior to measurable degradation. Addition of DTMAC to the same flask showed immediate degradation after a 2-week induction period. Samples from sites exposed to surfactants over prolonged periods, including a pond receiving direct discharge from a laundromat (13) and an activated sewage sludge from a municipal treatment plant (35), showed enhanced ability to utilize these agents as substrates. Shimp et al. (30) also studied the adaptation processes of bacterial communities to DTMAC. The ability of the community to degrade the surfactant increased 10- to 1000-fold after prolonged exposure. Interestingly, as long as the microbes were being exposed to minimal surfactant concentrations, adaptation and degradation continued; upon cessation of surfactant input, the ability to degrade DTMAC stopped.

The sorption of organic compounds to the clay and organic matter fractions of soils influences their potential for degradation by microorganisms. The bioavailability of a sorbed compound is dependent on the chemical and physical properties of the sorbate, the sorptive mechanism, the duration of sorbate-sorbent contact, and the properties and capabilities of the degradative organism(s). Many studies have shown that soil-sorbed compounds are unavailable to microbes. The decreased bioavailability in this state has been attributed to the

degree of adsorption (16, 33), entrapment in soil micropores (29, 34), soil organic matter content (40), rates of diffusion (17), nature of the sorbent (39), and the inability to scavenge by degradative organisms (18, 29). There are, however, exceptions to this generally accepted concept of reduced bioavailability of soil-sorbed substrates (18, 26, 31, 39). The exact mechanisms or processes aiding bioavailability have been the focus of much speculation, but little conclusive research has been completed.

The fate and persistence of a sorbed environmental contaminant are dependent, in part, on its availability to microbial degraders. Kinetics data concerning the biodegradation of NOCs and other compounds are useful in predicting remaining concentrations of recalcitrant compounds after a period of environmental exposure. This information can also be used to examine the bioavailability of soil- and sediment-sorbed substrates.

Miller and Alexander (28) developed a kinetic model to assess the bioavailability of clay-sorbed substrates based on adsorption isotherm data and the rate constant for biodegradation of the sorbate in the absence of sorbent. Additionally, the percentage of substrate converted to biomass and that fraction converted to CO_2 must be known or estimated in order to accurately model the system. The computational model is as follows:

$$\Delta S(t) = C(t)\ (1-e^{-k\Delta t}) \qquad (1)$$

where S is the substrate concentration and k is the first-order rate constant. The solution phase concentration, C, is obtained from the isotherm data and is initially estimated to be equal to S/2 by Newton's

iterative method. The model begins with the calculation of the total substrate remaining after t (accounting for incorporation into biomass), increases time by Δt and computes ΔS, updates the total remaining substrate concentration, and begins again until the desired time span is achieved. Reversible adsorption, fast desorption of sorbate, and degradation of only solution phase substrates are necessary to validate the model.

To describe the effects of the sorption of substrate and bacteria on the degradation of the sorbate, three kinetic models were proposed by Ogram et al. (29). These models incorporate the degradation and sorption relationships of both the substrate and the degradative bacteria. Assuming instantaneous equilibration and complete desorption of sorbed 2,4-D in soils, the data were modelled using a simple first-order production equation:

$$P = P_{max} (1 - e^{-kt}) \qquad (2)$$

where P = percentage of substrate mineralized to CO_2 over time, t. P_{max} refers to the maximal percentage of substrate mineralizable and k is a first-order rate constant. In this model, under the assumption that desorption is complete, P_{max} is set equal to 100% for both the soil-free and soil-containing systems. In doing so, the effects of sorption on the extent of substrate utilization cannot be determined.

Recently, Guerin and Boyd (18) developed a three parameter kinetic model to assess the bioavailability of sorbed substrates under non-equilibrium conditions:

$$P = v_2 t + [(v_1 + v_2)(1 - e^{-kt})]/k \qquad (3)$$

Here, P (percentage of added substrate mineralized) is related to v_1

(initial reaction rate), v_2 (mineralization rate due to the desorption of bound substrate), and k (the first-order rate constant) over time, t. In this coupled degradation-desorption model, v_2t describes the linear rate of sorbate desorption as solution phase substrate is removed through mineralization. In addition to providing information on the kinetics of sorbate desorption, the model describes the mineralization kinetics and quantity of readily available substrate. This new approach provides insight on the bioavailability of sorbed substrates with respect to differences in sorption coefficients, solute-sorbent aging, and microbial motility.

These and other models represent an attempt to assess the availability of soil- and sediment-sorbed substrates to microbial degraders based on the kinetics of mineralization in sorbent-free versus sorbent-containing systems. Compound hydrophobicity, release from a sorbed to a non-sorbed state, and soil particle size and aggregation regulate the bioavailability of a non-ionic organic compound. Substrate solubilization as a prerequisite for biodegradation will have many important implications in the bioremediation of groundwater and soil contaminated by NOCs.

REFERENCES

1. Blois, D.W. and J. Swarbrick. 1972. Interaction of quaternary ammonium bactericides with biological materials I: conductometric studies with cephalin. J. Pharmaceut. Sci. 61:390-399.

2. Boethling, R.S. 1984. Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants. Water Res. 18:1061-1076.

3. Boyd, S.A., W.F. Jaynes, and B.S. Ross. 1991. Immobilization of organic contaminants by organo-clays: Application to soil restoration and hazardous waste containment, pp. 181-200. *In* R.A. Baker (ed.), Organic Substances and Sediments in Water, vol. 1, Lewis Publishers, Chelsea, MI.

4. Boyd, S.A., J-F Lee, and M. Mortland. 1988. Attenuating organic compound mobility by soil modification. Nature. 333:345-347.

5. Boyd, S.A., Shaobai Sun, J-F Lee, and M. Mortland. 1988. Pentachlorophenol sorption by organo-clays. Clays Clay Miner. 36:125-130.

6. Briggs, G.G. 1969. Molecular structure of herbicides and their sorption by soils. Nature. 223:1288.

7. Burris, D.R. and C.P. Antworth. 1992. In situ modification of an aquifer by a cationic surfactant to enhance retardation of organic contaminants. J. Contam. Hydrol. 10:325-337.

8. Chiou, C.T., L.J. Peter, and V.H. Freed. 1979. A physical concept of soil-water equilibria for nonionic organic compounds. Science. 206:831-832.

9. Chiou, C.T., P.E. Porter, and D.W. Schmedding. 1983. Partition equilibria of nonionic organic compounds between soil organic matter and water. Environ. Sci. Technol. 17:227-231.

10. Chiou, C.T. and T.D. Shoup. Soil sorption of organic vapors and effects of humidity on sorptive mechanism and capacity. Environ. Sci. Technol. 19:1196-1200.

11. Dean-Raymond, D. and M. Alexander. 1977. Bacterial metabolism of quaternary ammonium compounds. Appl. Environ. Microbiol. 33:1037-1041.

12. Dubois, A.S. and E. Valko. 1945. Correlation between anti-bacterial power and chemical structures of higher alkylammonium ions. J. Bacteriol. 50:481-489.

13. Federle, T.W. and R.M. Ventullo. 1990. Mineralization of surfactants by the microbiota of submerged plant detritus. Appl. Environ. Microbiol. 56:333-339.

14. Garbarini, D.R. and L.W. Lion. 1986. Influence of the nature of soil organics on the soprtion of toluene and trichloroethylene. Environ. Sci. Technol. 20:1263-1269.

15. Gilbert, P. and A. Altaae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Letts. Appl. Microbiol. 1:101-104.

16. Gordon, A.S. and F.J. Millero. 1985. Adsorption mediated decrease in the biodegradation rate of organic compounds. Microbiol. Ecol. 11:289-295.

17. Greer, L.E. and D.R. Shelton. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-dichlorophenoxyacetic acid degradation in soil. Appl. Environ. Microbiol. 58:1459-1465.

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

19. Hansch, C. and J. Clayton. 1973. Lipophilic character and biological activity of drugs II: the parabolic case. J. Pharmaceut. Sci. 62:1-43.

20. Hassett, J.J., J.C. Means, W.J. Banwart, S.A. Wood, and A. Khan. 1980. Sorption of dibenzothiophene by soils and sediments. J. Environ. Qual. 9:184-186.

21. Isomaa, H., H. Bergman, and P. Sandberg. 1979. The binding of CTAB, a cationic surfactant to the rat erythrocyte membrane. Acta Pharmacol. et Toxicol. 44:36-42.

22. Jaynes, W.F. and S.A. Boyd. 1991. Clay mineral type and organic compound sorption by hexadecyltrimethylammonium-exchanged clays. Soil Sci. Soc. Amer. J. 55:43-48.

23. Karickhoff, S.W., D.S. Brown, and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 12:241-248.

24. Kondo, T. and M. Tomizawa. 1969. Mechanism of hemolysis by cationic surface active agents. J. Pharamaceut. Sci. 58:1378-1381.

25. Lambert, S.M. 1968. Omega, a useful index of soil sorption equilibria. J. Agr. Food Chem. 16:340-343.

26. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cationic surfactants in laboratory and environmental systems. Dev. Ind. Microbiol. 24:425-434.

27. Lee, J-F, J.R. Crum, and S.A. Boyd. 1989. Enhanced retention of organic contaminants by soils exchanged with organic cations. Environ. Sci. Technol. 23:1365-1372.

28. Miller, M.E. and M. Alexander. 1991. Kinetics of bacterial degradation of benzylamine in a montmorillonite suspension. Environ. Sci. Technol. 25:240-245.

29. Ogram, A.V., R.E. Jessup, L.T. Ou, and R.S.C. Rao. 1985. Effects of sorption on biological degradation rates of (2,4-Dichlorophenoxy) acetic acid in soils. Appl. Environ. Microbiol. 49:582-587.

30. Shimp, R.J., B.S. Schwab, and R.J. Larson. 1989. Adaptation to a quaternary ammonium surfactant by suspended microbial communities in a model stream. Environ. Toxicol. Chem. 8:723-730.

31. Shimp, R. J. and R.L. Young. 1988. Availability of organic chemicals for biodegradation in settled bottom sediments. Ecotoxicol. Environ. Safety. 15:31-45.

32. Sigoillet, J-C and M-H Nguyen. 1990. Isolation and characterization of surfactant-degrading bacteria in a marine environment. FEMS Microbiol. Ecol. 73:59-68.

33. Steen, W.C., D.F. Paris, and G.L. Baughman. 1980. Effects of sediment sorption on microbial degradation of toxic substances, pp. 477-482. *In* R.A. Baker (ed.), Contaminants and Sediments. Vol. 1. Ann Arbor Science, Ann Arbor, MI.

34. 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. 21:1201-1208.

35. Sullivan, D.E. 1983. Biodegradation of a cationic surfactant in activated sludge. Water Res. 17:1145-1151.

36. Tubbing, D.M.J. and W. Admiraal. 1991. Inhibition of bacterial and phytoplanktonic metabolic activity in the lower Rhine River by ditallowdimethylammonium chloride. Appl. Environ. Microbiol. 57:3616-3622.

37. van Ginkel, C.G., J.B. van Dijk and A.G.M. Kroon. 1992. Metabolism of hexadecyltrimethylammonium in *Pseudomonas* strain B1. Appl. Environ. Microbiol. 58:3083-3087.

38. Ventullo, R.M. and R.J. Larson. 1986. Adaptation of aquatic microbial communities to quaternary ammonium compounds. Appl. Environ. Microbiol. 51-356-361.

39. Weber, J.B. and H.D. Coble. 1968. Microbial decomposition of diquat adsorbed on montmorillonite and kaolinite clays. J. Agr. Food Chem. 16:475-478.

40. Weissenfels, W.D. H-J Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl. Microbiol. Biotechnol. 36:689-696.

CHAPTER 1

BIOSTABILITY OF SOIL- AND CLAY-EXCHANGED HEXADECYLTRIMETHYLAMMONIUM

INTRODUCTION.

The soil organic matter (SOM) fraction of soils plays a predominant role in the sorption of non-ionic organic contaminants (NOCs) from water. Non-polar interactions between solute molecules and the SOM result in NOC partitioning into amorphous SOM (5, 6). Mineral phases in soils are generally ineffective as sorbents of NOCs due to the polar nature of minerals such as silica and metal oxides, and the presence of hydrated exchange ions such as Na^+, K^+, Mg^{++}, and Ca^{++} on clays (7). Therefore, low organic matter surface soils and subsoils have a limited capability to sorb NOCs, increasing the potential for NOC transport in the soil profile.

Recently, we have developed a soil modification approach for reducing contaminant transport in soils, subsoils, and aquifer materials. Our studies have shown that the sorptive capacity of soils and subsoils can be greatly increased through the addition of large hydrophobic organic cations of the general form $[(CH_3)_3NR]^+$ or $[(CH_3)_2NRR']^+$, where R and R' are large alkyl hydrocarbons, as for example hexadecyltrimethylammonium (HDTMA). Once added to soil, the organic cations displace native inorganic cations from the cation exchange sites of soil clays resulting in the formation of an organic partition phase comprised of the alkyl hydrocarbon groups (e.g., the C-16 groups of HDTMA) of the organic cation. Lee et al. (16) demonstrated

that the sorption coefficients (K values) of several aromatic hydrocarbons (e.g., benzene, toluene, and ethylbenzene) could be increased by over two orders of magnitude in B-horizon subsoils treated with HDTMA in an amount equivalent to the cation exchange capacity (CEC) of the soil. Increases in K values of this magnitude substantially decrease the mobility and leaching potential of organic contaminants, such as benzene, that are commonly found in groundwater. The organic sorptive phase formed in the HDTMA-treated soils studied by Lee et al. (16) was 10 to 30 times more effective on a unit mass basis than natural SOM in removing NOCs from solution. HDTMA forms a low polarity organic phase derived from the interacting C-16 hydrocarbon tails, whereas SOM contains more polar groups which lowers its effectiveness as a partition phase for NOCs.

Remediation of a polluted site could be accomplished by coupling this immobilization technology with *in situ* bioremediation of the NOCs. *In situ* formation of organo-clay complexes from naturally-occurring clays (i.e., those present in subsoils or aquifer materials) could be achieved by injection of HDTMA into the subsurface to create a sorptive zone that would retard NOC migration and concentrate target contaminants within a confined area (1). Burris and Antworth (4) have demonstrated the feasibility of this concept by injecting HDTMA into confined aquifer material; the resultant sorptive barrier effectively immobilized naphthalene in a simulated contaminant plume. Subsequent biodegradation of the immobilized contaminants would constitute a comprehensive soil restoration technology.

Organo-clays may also be employed as an additional containment (sorptive) zone around hazardous waste containment facilities or chemical storage tanks which currently use bentonite liners as a physical barrier to reduce hydraulic conductivity (2). In such instances, when inadvertently released NOCs make contact with the modified clay layer, sorption and immobilization will halt further contaminant migration and ultimately reduce the extent of contamination.

An important consideration in the development of these applications is the stability of the soil- or clay-bound quaternary ammonium compound (QUAT) to microbial attack, either directly on the mineral surface or by extracellular enzymes which may penetrate the interlamellar layers of the clay structure. Although certain QUATs are used as anti-microbial agents (10, 15), microbial adaptation to and mineralization of QUATs have been observed (8, 9, 14, 20, 21, 22, 23). Therefore, it is important to evaluate the biostability of HDTMA exchanged onto soils and clay mineral components of soils.

In this study, HDTMA-exchanged organic complexes were prepared from several clay minerals (illite, smectite, and vermiculite) and soils. The biostability of these complexes were evaluated under saturated and unsaturated conditions in a variety of soils and in pure cultures of HDTMA-degrading microorganisms that we isolated.

MATERIALS AND METHODS.

Collection of soils. Marlette (Fine-loamy, mixed, mesic Glossoboric Hapludalfs) A horizon (sandy loam) and B horizon (loam) and Spinks (Sandy, mixed, mesic Psammentic Hapludalfs) A horizon (loamy sand) and B horizon (sandy loam) soils were collected from agricultural fields near East Lansing, MI. An additional soil was obtained from northern California which contained approximately 500 ug L^{-1} total petroleum hydrocarbons following a bioremediation clean-up utilizing anionic surfactants. After collection, the soils were briefly air dried (1 hour), screened to pass a 2 mm sieve, and placed in refrigerated storage at 4 °C. Soils were characterized with respect to cation exchange capacity (17), organic carbon content (Dohrmann DC-190 Organic Carbon Analyzer, Rosemount Analytical, Inc., Santa Clara, CA), particle size distribution analysis by the hydrometer method (11), and field capacity (18). The soil properties are summarized in Table 1.1.

Preparation of Sample Clays. The following clays were used in this study: Mg-saturated smectite from Crook County, Wyoming (SWy), Na-saturated vermiculite from South Carolina (VSC), and Mg-saturated illite from Silver Hill, Montana (IMt) having cation exchange capacities (CECs) of 90, 80, and 24 meq/100 g, respectively (13). Organo-clay complexes were prepared by reacting 10 g of the < 2 um size fraction of each clay with HDTMA-Bromide. A mixture of ^{14}C-HDTMA and unlabelled HDTMA was dissolved in Milli-Q grade water and added in an amount equal to 30, 70, or 100% of the CEC of the clay. ^{14}C-HDTMA was added to achieve a total activity of 0.5 uCi g^{-1} of clay. The mixtures were allowed to equilibrate for one hour while continuously stirring. The prepared

TABLE 1.1. Physical and Chemical Characteristics of Soils and Clays used in this study.

Soil	% sand	% silt	% clay	Field Capacity (mL/100 grams)	CEC (meq/100 grams)	% Organic Carbon
Marlette A	54.9	35.7	9.4	29.2	11.5	2.2
Marlette B	51.5	31.3	17.2	33.9	12.8	0.6
Spinks A	75.6	19.6	4.8	19.3	5.9	1.5
Spinks B	60.2	27.2	12.6	28.6	8.9	0.3
Petroleum-Contaminated	52.0	11.0	29.0	5.1		1.9

clays were collected by centrifugation (9,000 x *g*, 15 minutes) and rinsed in centrifuge tubes four times with doubly deionized water to remove residual HDTMA. Then, the exchanged clays were freeze-dried and stored at room temperature. To determine the specific activity of the clays, 20 mg portions of each sample were combusted in a Roboprep-CN Biological Sample Converter (Europa Scientific, U.K.) and the collected $^{14}CO_2$ was analyzed by liquid scintillation counting (LSC) on a Packard 1500 TriCarb Liquid Scintillation Analyzer (Packard Instrument Co., Downer's Grove, IL). The radioactivity was corrected for background levels after being converted to disintegrations per minute (dpm) using external standard quench correction.

Preparation of HDTMA-soils. Two HDTMA-modified soils were prepared using Marlette soil A and B horizons. Ten grams of soil were added to beakers containing 25 mL of Milli-Q grade water. ^{14}C-labelled HDTMA solutions were prepared which would satisfy 70% of the CECs of the individual soils and achieve an activity of 0.5 uCi g^{-1}. These solutions were added to the soil slurries while stirring continuously, and the systems were equilibrated for 4 hours. The soils were then collected by centrifugation (8000 x *g*, 15 minutes), washed once with doubly deionized water, and allowed to air dry before storage at room temperature. 20 mg samples of each organo-soil were analyzed for total ^{14}C-activity using an oxidation chamber and a liquid scintillation analyzer as described above.

Chemicals. Hexadecyltrimethylammonium bromide, 99% purity, was obtained from Sigma Chemical Co., St. Louis, MO. ^{14}C-HDTMA-Br, labelled on the terminal carbon of the hexadecyl chain, was obtained from Moravek

Biochemicals, Inc. (Brea, CA); it had a specific activity of 55 mCi $mmol^{-1}$ and a radiochemical purity of > 98%. The molecular structure of HDTMA is shown in the Appendix. All chemicals were used without further purification.

Isolation of HDTMA-degrading microbes. Two HDTMA-degrading organisms were used in these studies. The two isolates, designated 101S and 102G, were isolated from an activated sludge sample obtained from the Lansing, Michigan municipal waste water treatment plant. An aliquot of the sludge was introduced into three flasks containing a minimal medium [high-buffer broth (HBB)(12)] along with n-hexadecane (442 uM) and HDTMA at varying concentrations (10 uM to 1 mM). After an initial increase in turbidity, aliquots of the growing cultures were consecutively transferred (three times) to fresh HBB containing n-hexadecane and HDTMA. Following the transfers, an aliquot was inoculated into HBB containing HDTMA as the sole carbon source. Upon an increase in turbidity, the culture was plated onto one-half strength nutrient agar and isolated colonies were reinoculated into HBB/HDTMA. Characterization of the two isolates is in progress.

Biostability of HDTMA-clays in Soils.

(1) Incubation in Marlette A, B and Spinks A, B horizons:

The long-term biostability of HDTMA-illite, -smectite, and -vermiculite was assessed through several experiments which employed incubation lengths of 12 and 18 months. In the experimental procedure, 25 g (dry weight basis) of each soil were separately placed in 125 mL Erlenmeyer flasks along with 100 mg of either HDTMA-illite, -smectite, or -vermiculite prepared at 100% of their CECs. The soil and clay were

then thoroughly mixed and brought to 70% of the field capacity of the soil through the addition of deionized water. Additionally, duplicate flasks containing 25 g of one of the four soils (no HDTMA-clay added) were amended with HDTMA added directly as the free cation in an amount equal to the calculated amount of HDTMA added to the HDTMA-smectite amended soils. For this addition, a mixture of ^{14}C-HDTMA and unlabelled HDTMA was added as an aqueous solution. A 1 mL aliquot of the solution was analyzed by liquid scintillation counting to determine the radioactivity added to each soil. All flasks were capped with a # 5 rubber stopper equipped with a suspended glass vial containing 1 mL of a 2 N solution of KOH used to trap evolved $^{14}CO_2$, and incubated at 25 °C. Periodically, the flasks were opened and the KOH was transferred to glass scintillation vials containing 7.5 mL of scintillation counting cocktail for aqueous solutions along with 3 mL of 95% ethanol used to rinse the glass vial. The KOH was replaced with a fresh solution. Radioactivity was counted after the samples had been stored in the dark for at least 24 hours to reduce chemiluminescence.

A similar experiment was set up to study the long-term biostability of the HDTMA-exchanged clays at levels equal to or below their CECs using all three of the organo-clays prepared at 30, 70, and 100% of their CECs. All experimental parameters were identical to those previously described.

(2) Incubation in a Petroleum Hydrocarbon Contaminated (PHC) Soil:

The experimental design was similar to that previously described for the Marlette and Spinks soils. Duplicate flasks were set up which contained 25 g of the petroleum hydrocarbon-containing soil along with

100 mg of either HDTMA-illite, -smectite or -vermiculite prepared at 100% of the clay's CEC. The soil was wetted to 70% of field capacity and thoroughly mixed. Additionally, two flasks containing only the soil (no HDTMA-clay added) were amended with HDTMA added as an aqueous solution as described above. All experimental sampling procedures were identical to those previously mentioned.

(3) Incubation in a Marlette A Horizon Soil Slurry:

A soil slurry system was designed to measure the stability of the organo-clays under saturated conditions. Experiments were conducted using 125 mL Erlenmeyer flasks equipped with a rubber stopper and a suspended vial containing 1 mL of a 2 N KOH solution as previously described. All flasks were incubated at 25 °C on a rotary shaker (150 rpm). The experimental procedure involved the addition of 50 mL of deionized water to 25 grams of Marlette soil from the A horizon. 100 mg of either HDTMA-illite, -smectite, or -vermiculite, prepared by adding HDTMA at 100% of the CEC of the clay, were added to duplicate flasks. Two additional soil slurry flasks received HDTMA added directly as the free cation in an aqueous solution of unlabelled and ^{14}C-labelled HDTMA. Initial activity of the HDTMA solution was measured using LSC prior to its addition to the soil. The flasks were periodically opened and the KOH was removed and replaced with fresh alkali. The KOH containing the $^{14}CO_2$ was then analyzed by LSC and the total $^{14}CO_2$ evolved was determined. The experiment was terminated after 19 weeks.

(4) Incubation in a Petroleum Hydrocarbon Contaminated Soil Slurry

Experimental apparatus and parameters were identical to those employed in the Marlette A horizon soil slurries.

Biostability of HDTMA-Clays in Cultures of HDTMA-Degraders. Doubly deionized water was used to prepare a mineral medium (NM1) which contained (g/L): $NaNO_3$ (3.0), NaH_2PO_4 (0.5), Na_2HPO_4 (0.5), NaCl (0.5), 1 mL of trace elements (19), 1 mL of ferric quinate (0.27 g $FeCl_3$ and 0.19 g quinic acid per 100 mL water) and 1 mL of a vitamin solution (17); it was adjusted to a pH of 7.00 with 2 N HCl. 100 mg of either HDTMA-illite, -smectite, or -vermiculite, prepared at 30, 70, or 100% of their respective CECs, were added to serum bottles containing 75 mL of the nutrient media. An inoculum of HDTMA-degrading bacteria (designated 101S) was standardized by measurements of optical density at 600 nm to achieve a final cell density of 10^8 cells mL^{-1}. Clay-free "control" bottles contained HDTMA in an amount equal to that bound to the smectite complexes. Serum bottles were crimp-capped and incubated at 25 °C on a rotary shaker (150 rpm). At predetermined intervals, 1 mL of the aqueous phase and 1 mL of the gaseous phase in the serum bottles were removed by syringe, transferred to a tube containing 1 mL 2 N HCl, and the evolved $^{14}CO_2$ was trapped on KOH-saturated filter paper suspended from the stopper in a plastic cup. Tubes were allowed to sit for 24 hours to allow quantitative recovery of $^{14}CO_2$. The filter paper was transferred to glass scintillation vials containing 7.5 mL of scintillation cocktail along with 3 mL of ethanol used to rinse the cup. The samples were counted by LSC and the total radioactivity evolved was determined.

X-ray Diffraction Analysis. The HDTMA-clays were subjected to two different treatments prior to x-ray diffraction analysis. HDTMA desorption studies were conducted in 25 mL Teflon centrifuge tubes by

equilibrating 100 mg of each HDTMA-clay with 25 mL of the sterile nutrient media for 24 hours on a rotary shaker (20 rpm). The tubes were centrifuged (8000 x *g*, 10 minutes) and 1 mL of the supernatant was transferred to scintillation cocktail for counting. The supernatant was decanted and replaced with 25 mL of fresh media. After nine consecutive washes, the clay was collected, rinsed, and resuspended in ethanol.

The second treatment involved the incubation of the HDTMA-clays with an HDTMA-degrading culture of bacteria (101S). 33 mg of organo-clay were weighed into 150 mL serum bottles containing 75 mL of nutrient media and 10^8 cells mL^{-1} of 101S. The bottles were crimp-capped and incubated at 25 °C on a rotary shaker (150 rpm). At predetermined intervals over a five-day period, 1 mL aliquots of both the aqueous and gaseous phases were removed by syringe and transferred to the previously described $^{14}CO_2$ trap. Mineralization of the available HDTMA was considered complete after the 5-day incubation. At that point, the clays were allowed to settle, were collected with a Pasteur pipet, and were transferred to Nalgene centrifuge tubes where they were rinsed once with 95% ethanol and collected by centrifugation (8000 x *g*, 15 minutes). The HDTMA-clays were then dispersed in ethanol. A control sample of each clay was prepared by dispersing 50 mg of the HDTMA-clay that had not been incubated with 101S or subjected to the desorption process in 0.5 mL of 95% ethanol. Samples from all three treatments were dried as aggregates on glass slides and the basal X-ray diffraction spacings were then recorded by a Phillips APD 3720 automated X-ray diffractometer (Phillips Electronic Instruments, Mahwah, NJ).

Biostability of HDTMA-Soils.

(1) Incubation in a Culture of an HDTMA-degrader:

The ^{14}C-labelled HDTMA-Marlette A and HDTMA-Marlette B horizon soils were incubated in 75 mL of nutrient media containing a pure culture of 102G (10^8 cells mL^{-1}) to assess the biostability of HDTMA when bound primarily to the organic matter (A horizon soil) versus the mineral matter (B horizon soil) of soils. Sampling and analysis procedures are identical to those previously described for such assays.

(2) Desorption of HDTMA in HDTMA-soils:

The percentage of bound HDTMA that was exchangeable for ions in the nutrient media was determined in the following assay. 100 mg of the organo soil were placed into a 25 mL centrifuge tube along with 25 mL of the media and the system was allowed to equilibrate 24 hours at 20 rpm on a rotator. The tubes were then centrifuged (8000 x *g*, 10 minutes) and 1 mL of the supernatant was transferred to scintillation cocktail for counting the radioactivity. The remainder was decanted and replaced with fresh sterile media. This process was continued for nine consecutive washes.

Mass Balance Determination. At the termination of the ^{14}C-HDTMA-clay incubations in Marlette A and B horizon and Spinks A and B horizon soils at 70% field capacity, representative flasks were analyzed to attempt a mass balance determination of the ^{14}C-HDTMA remaining in the soil and the $^{14}CO_2$ evolved over the course of the assay. Soils were air dried and homogenized before combustion of three 0.5-gram portions in a Harvey Biological Material Oxidizer model OX-300 (R.J. Harvey Instrument Corporation). $^{14}CO_2$ was collected and analyzed by LSC to determine the

total ^{14}C-HDTMA remaining in the soil. Knowing the initial ^{14}C-HDTMA added and the total $^{14}CO_2$ collected over the twelve month incubation, the results of the oxidation analysis were used to attempt a mass balance by summing the ^{14}C-HDTMA remaining in the soil and that evolved as $^{14}CO_2$.

RESULTS.

The results from the long-term incubations in Marlette and Spinks A and B horizon soils, designed to test the biostability of HDTMA-exchanged clays are summarized in Table 1.2 and Figures 1.1-1.6. Occasionally, one of the duplicate treatments mineralized significantly greater amounts of HDTMA than the corresponding replicate, and these values are presented individually in Table 1.2 and Figures 1.1-1.6 when the variation was greater than 25%. Most of the HDTMA mineralization curves were indicative of first-order kinetics, however, some replicates experienced a lag of up to nine months before significant mineralization commenced.

In general, HDTMA added free or clay-bound was resistant to biodegradation in both the A and B horizons of two representative agricultural soils (Marlette and Spinks). Less than 5% of the added HDTMA was recovered as CO_2 in 85% of the 128 biometer flasks used in the Marlette and Spinks soil incubations (Figure 1.7). Mass balance calculations were performed to determine the effectiveness of the $^{14}CO_2$ traps. In flasks producing significant quantities (> 20%) of $^{14}CO_2$, approximately two-thirds of the added ^{14}C-HDTMA could be accounted for, indicating loss of $^{14}CO_2$ to the atmosphere during biometer sampling. Recoveries of > 99% of the added ^{14}C-HDTMA were observed in flasks which showed no significant (< 1%) mineralization of HDTMA. While mass balances were not performed on samples producing intermediate amounts of CO_2 ($1\% < x < 20\%$), these values probably represent the upper and lower bound of recoveries in the experimental system.

Table 1.2. Cumulative % CO_2 recoveries[a] from the mineralization of HDTMA added to soil directly as the free cation or as a HDTMA-clay.

SOIL TYPE	HDTMA LOADING (%CEC OF CLAY)	FREE-HDTMA[b]	HDTMA-ILLITE	HDTMA-SMECTITE	HDTMA-VERMICULITE
Marlette A (Sandy Loam)	30	2.0, 33.9	3.3	<1.0, 2.1	2.7
	70	1.7	3.4	<1.0	<1.0, 30.0
	100	1.3	2.2	<1, 11.9	<1.0
	100[c]	1.0, 7.1	3.5, 29.4	2.1	1.1, 2.9
Marlette B (Loam)	30	<1.0	<1.0, 1.3	<1.0	<1.0
	70	<1.0	1.0	<1.0	<1.0
	100	<1.0	<1.0, 8.3	<1.0	<1.0
	100[c]	<1.0	1.0, 4.5	<1.0	1.0. 30.2
Spinks A (Loamy Sand)	30	4.9, 31.3	6.3	1.1	3.7
	70	3.6, 36.1	5.4	2.1, 4.6	2.3, 24.1
	100	2.0, 43.6	3.9	2.0	1.5
	100[c]	1.1	2.7	1.9, 20.4	1.8, 2.5
Spinks B (Sandy Loam)	30	<1.0	<1.0, 37.6	<1.0, 12.6	1.1
	70	<1.0	1.4	<1.0	<1.0
	100	<1.0	<1.0	<1.0	<1.0, 36.1
	100[c]	1.0	1.3	1.6	1.0

[a] Single values reported are averages of duplicate measurements. When the duplicates differed by > 25%, the individual values are reported.

[b] HDTMA was added directly to soil in an amount equivalent to that added in the HDTMA-smectite treatment.

[c] Denotes incubation time of 18 months at 100% of the CEC. All other values are at 12 months.

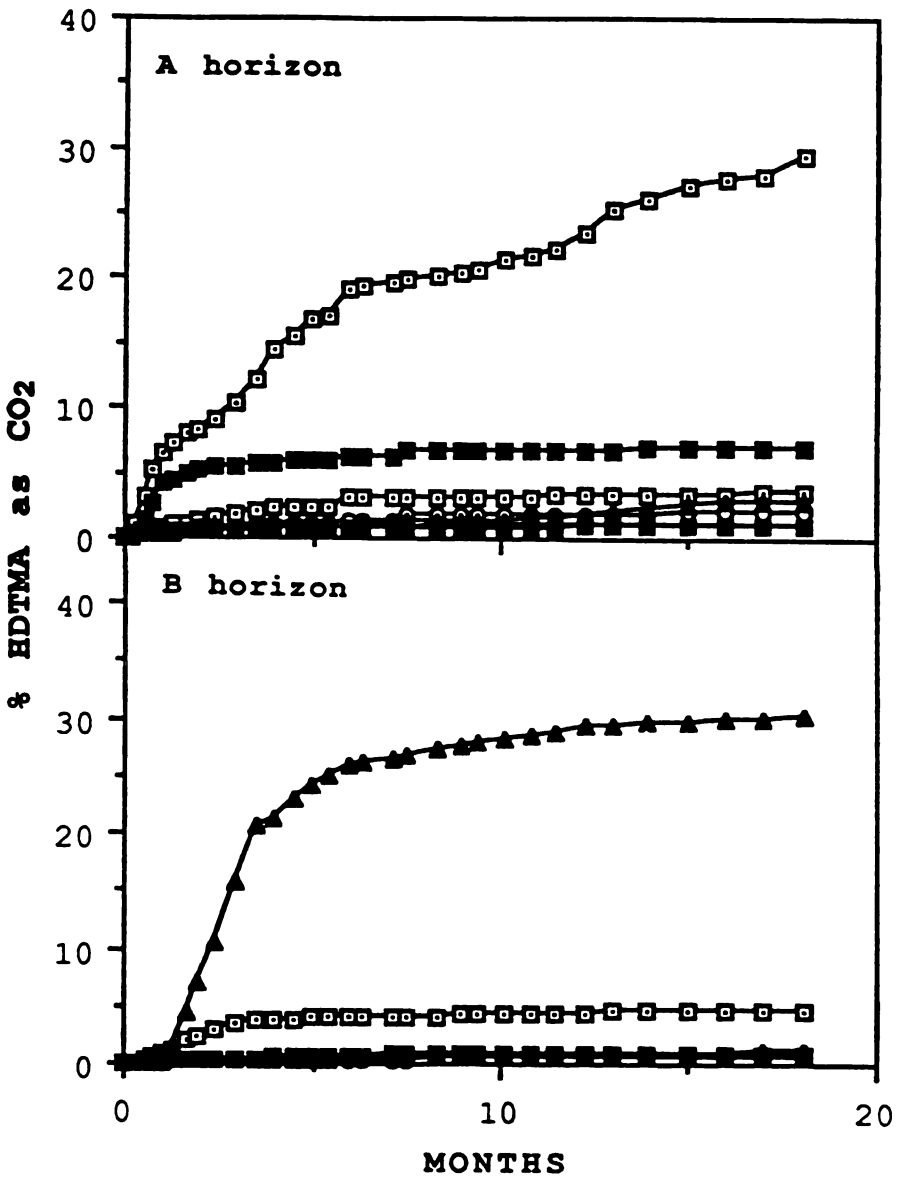

Figure 1.1. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay (100% CEC) to the Marlette soil. (■ free HDTMA, ⊡ illite, ○ smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

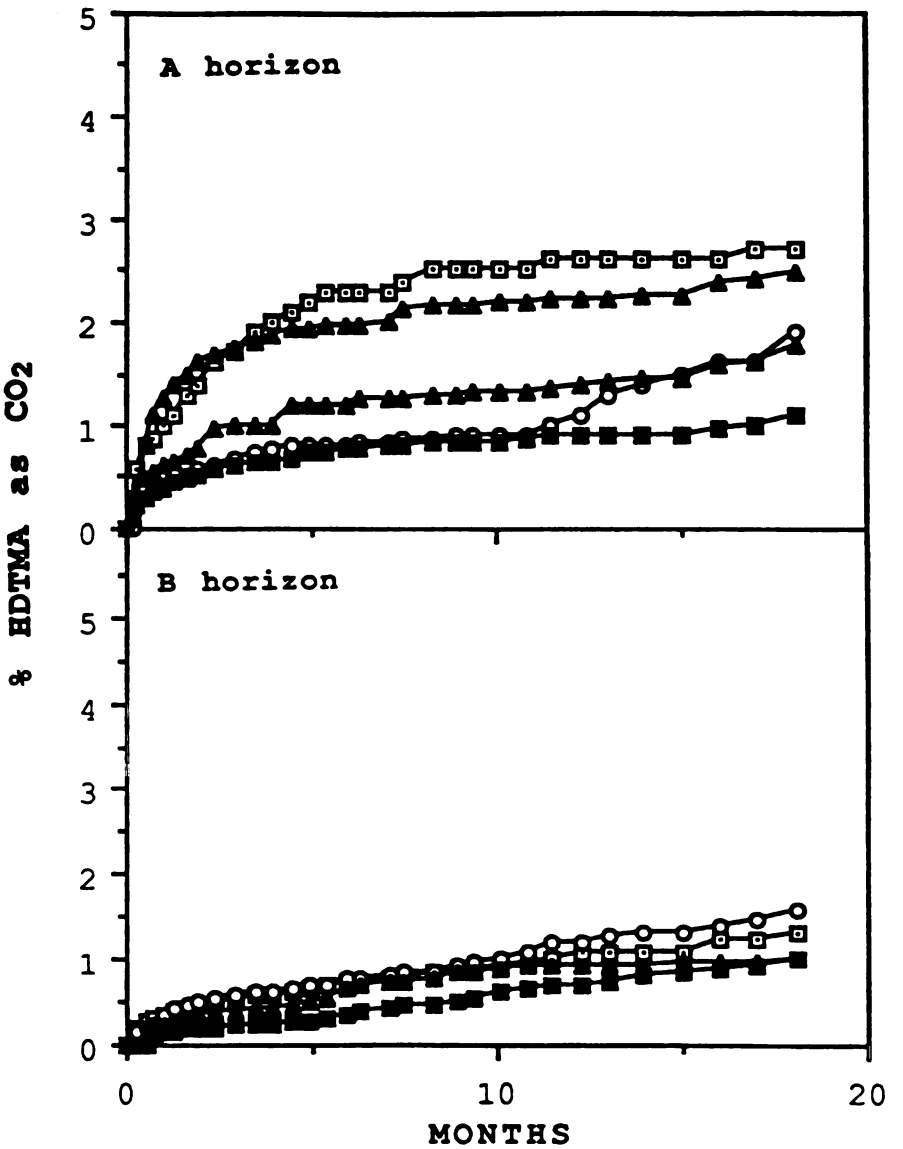

Figure 1.2. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay (100% CEC) to the Spinks soil. (■ free HDTMA, ⊡ illite, ○ smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

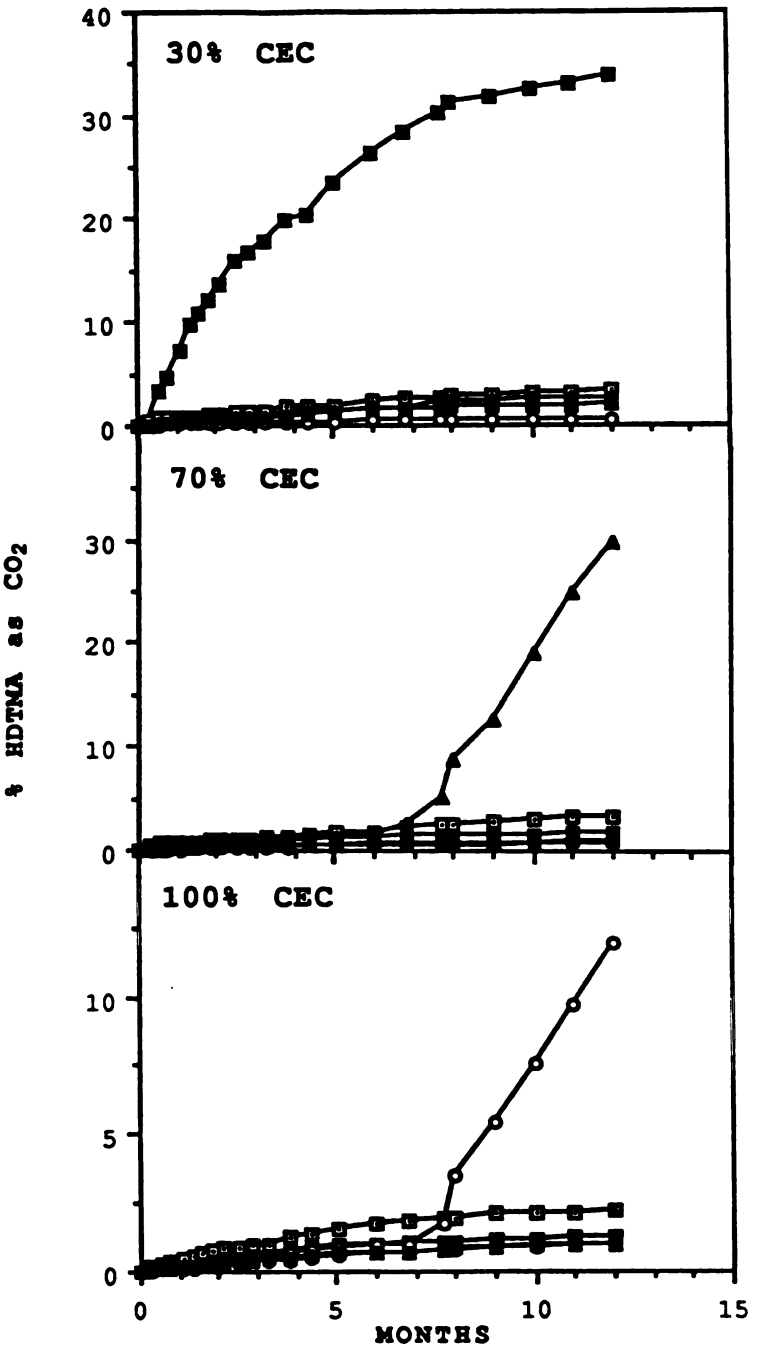

Figure 1.3. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay to the Marlette A horizon soil. (■ free HDTMA, ⊡ illite, ● smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

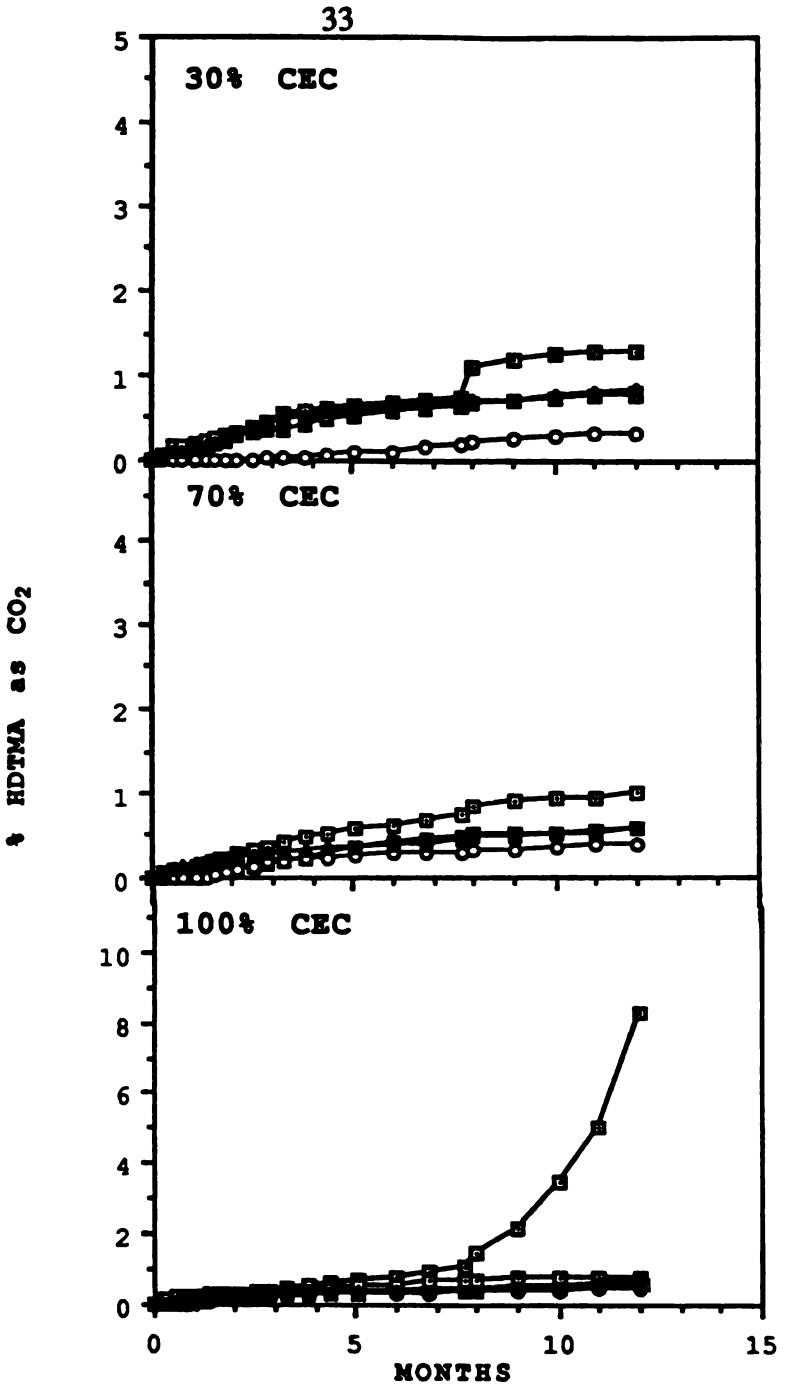

Figure 1.4. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay to the Marlette B horizon soil. (■ free HDTMA, ⊡ illite, O smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

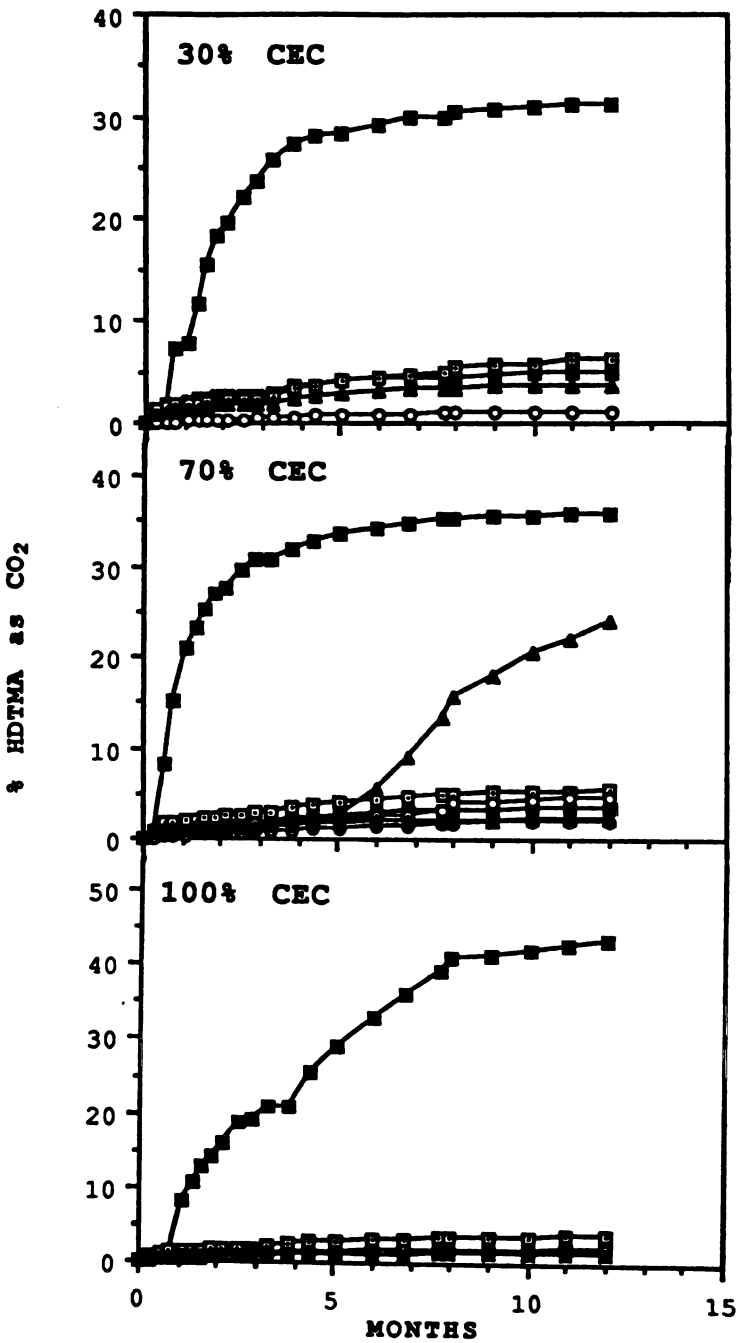

Figure 1.5. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay to the Spinks A horizon soil. (■ free HDTMA, ⊡ illite, ○ smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

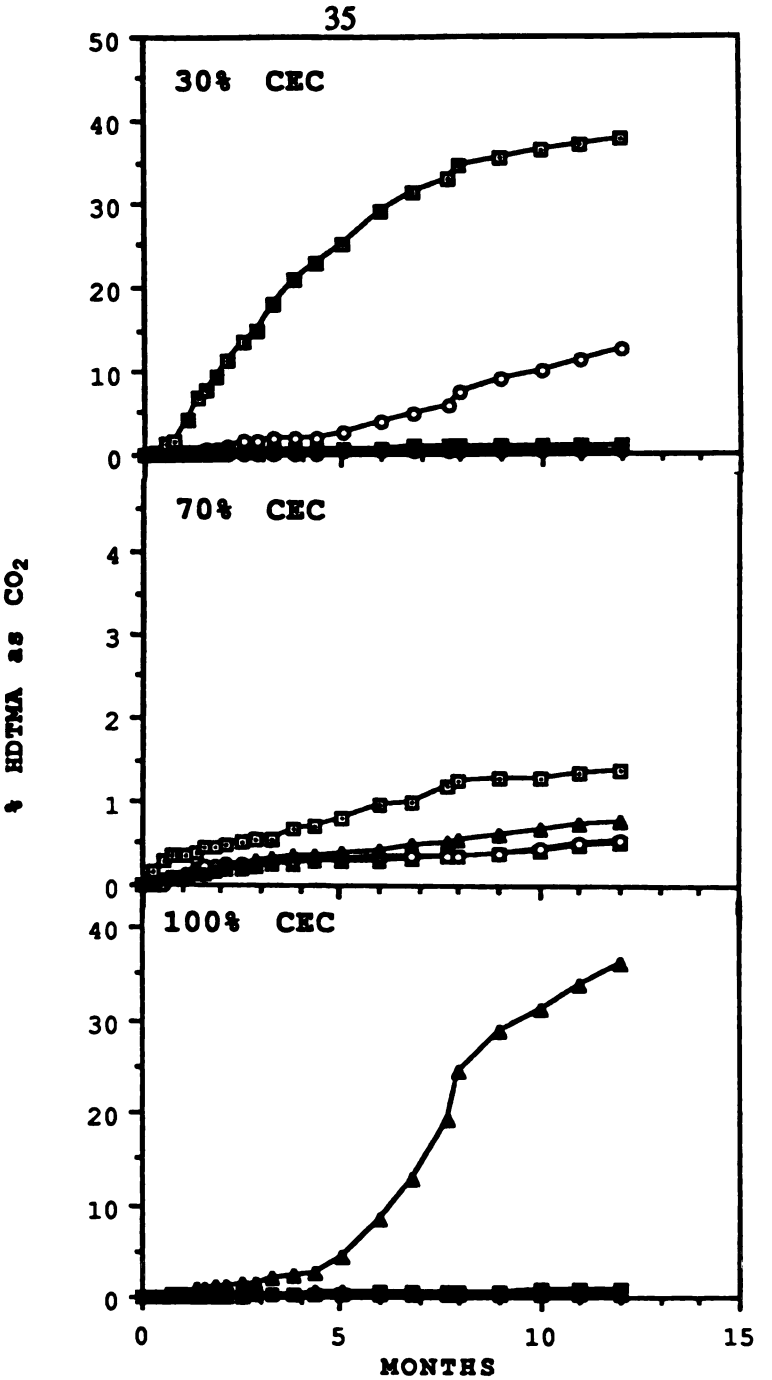

Figure 1.6. Progress curves showing the cumulative CO_2 recovery from mineralized HDTMA added directly as the free cation or as a HDTMA-clay to the Spinks B horizon soil. (■ free HDTMA, ⊡ illite, o smectite, ▲ vermiculite.) The data presented are averages of duplicate values except when the final cumulative CO_2 values differed by more than 25%; then the individual replicates are plotted.

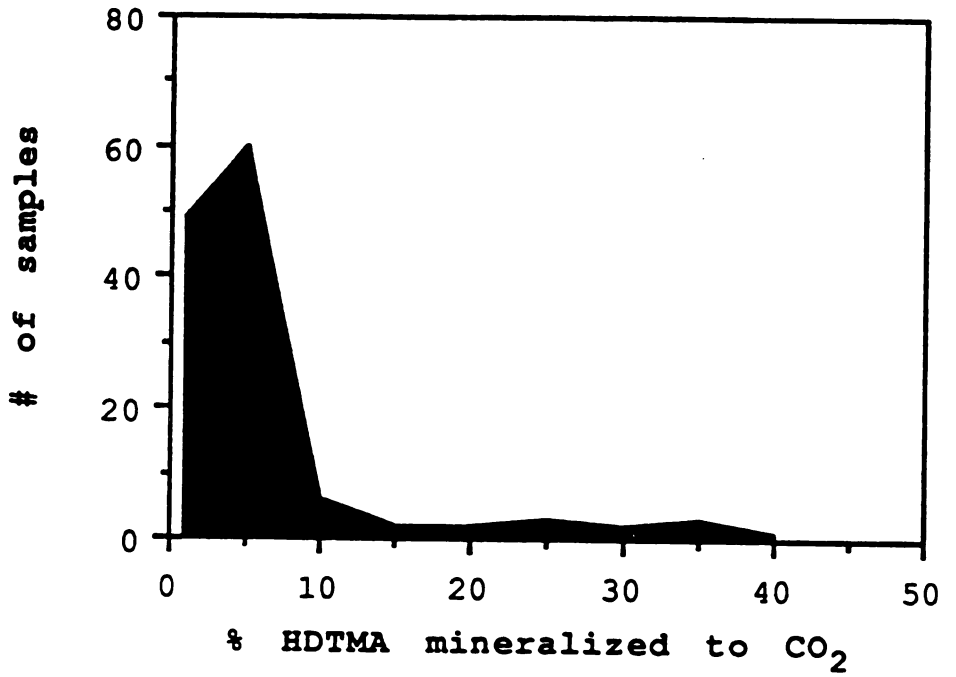

FIGURE 1.7. Schematic representing overall biostability of HDTMA and HDTMA-clays in soils.

Incubations were also carried out using a petroleum-contaminated soil to evaluate the biostability of HDTMA in a soil that should have an active population of hydrocarbon-degrading bacteria. In this soil, which was moistened to 70% of field capacity, less than 7% of the HDTMA was mineralized when added freely or bound to smectite or vermiculite. HDTMA-illite, however, was less stable and replicate flasks evolved 14.9 and 25% of the HDTMA as CO_2 after a 6-month incubation (Table 1.3).

Slurry experiments using both the Marlette A horizon soil and PHC-soil were designed to assess the effects of water saturation on the biostability of HDTMA and HDTMA-clays in soil systems (Table 1.3). Greater stability was observed in these systems as compared to incubations at levels below saturation, (e.g., 70% of field capacity) (Table 1.2). In the PHC-soil slurry, 7 of 8 biometers mineralized less than 5% of the added HDTMA completely to CO_2 over a 6-month period. In the Marlette A horizon soil slurries, very little mineralization (< 2%) occurred over a five-month incubation (Table 1.3).

The percentage of HDTMA mineralized when added in an unbound form in both the Marlette and Spinks A horizon soils was consistently greater than for any of the HDTMA-clay complexes tested. In B horizon soils, the HDTMA added in the unbound form was very stable (1% or less was recovered as CO_2). HDTMA-clay complexes added to subsurface soils were also more biologically stable than when added to surface soils.

The stability of HDTMA complexes with illite, smectite, and vermiculite were compared. HDTMA-smectite complexes offer the most resistance to microbial degradation, (i.e., have the greatest biostability). Less than 1% of the HDTMA in HDTMA-smectite was

Table 1.3. Cumulative % CO_2 recoveries[a] from the mineralization of HDTMA added to soil directly as the free cation or as a HDTMA-clay.

SOIL TYPE	HDTMA LOADING (%CEC OF CLAY)	FREE-HDTMA[b]	HDTMA-ILLITE	HDTMA-SMECTITE	HDTMA-VERMICULITE
PHC[c]	100	6.2	14.9, 25.0	6.4	7.0
PHC-Slurry[d]	100	1.1, 2.0	<1.0, 1.5	<1.0, 2.8	4.9, 19.5
Marlette A-Slurry[e]	100	<1.0	1.9	<1.0	<1.0

a Single values reported are averages of duplicate measurements. When the duplicates differed by > 25%, the individual values are reported.
b HDTMA was added directly to soil in an amount equivalent to that added in the HDTMA-smectite treatment.
c PHC = petroleum hydrocarbon contaminated, experiment terminated at 6.2 months.
d Experiment terminated at 5.3 months.
e Experiment terminated at 4.8 months.

mineralized in more than half of the biometers containing the organo-clay and an agricultural soil. The HDTMA-vermiculite complexes proved to be less biologically stable than the smectite complexes, and HDTMA-illite was the most susceptible to degradation by indigenous soil microbes. In the PHC-soil, only 6.4% of the added HDTMA bound to smectite was recovered as CO_2 despite prior exposure to a surfactant and the high population of petroleum-degrading microbes (10^5 to 10^6 degraders/g soil).

Two HDTMA-degrading bacteria (designated 101S and 102G) were isolated for use in additional biostability experiments. These isolates carried out complete mineralization of HDTMA to CO_2 plus biomass in a ratio of approximately 3:2 at a cell density of 10^8 cells mL^{-1}. First-order mineralization kinetics were observed at this cell density and the results of the assay utilizing 101S are presented in Table 1.4.

Table 1.4. Mineralization of HDTMA and HDTMA-clays by organism 101S.

% CEC loading	% ^{14}C-HDTMA Recovered as $^{14}CO_2$			
	Free HDTMA	Illite	Smectite	Vermiculite
30	57.7	59.7	4.7	42.1
70	57.3	61.8	9.9	36.8
100	48.0	65.9	12.4	35.1

Under these rigorous experimental conditions involving inoculation with a high density of HDTMA-utilizers, HDTMA-smectite complexes prepared at all three levels of CEC saturation, were fairly resistant; only 4.7 to 12.4% of the HDTMA was evolved as CO_2. HDTMA, bound to illite and

vermiculite was degraded to a much larger extent. In the case of HDTMA-illite, given the ratio of HDTMA converted to biomass and CO_2, it is possible that practically all of the exchanged cation was mineralized.

A desorption study was performed to determine if the clay-bound HDTMA was being desorbed by the nutrient media employed in the mineralization assays. After nine consecutive washes of the prepared organo-clays in the nutrient media, it was found that very little of the bound HDTMA easily desorbs (Table 1.5).

Table 1.5. Cumulative percentage of HDTMA desorbed from HDTMA-clays in nine consecutive batch desorption steps using basal salts media.

% CEC loading	Illite	Smectite	Vermiculite
30	3.2	1.3	6.5
70	6.4	1.2	5.0
100	9.2	2.9	9.0

Desorption of HDTMA from HDTMA-smectite was lower (1 to 3%) than from HDTMA-illite or -vermiculite (3 to 9%).

Basal x-ray diffraction spacings of all three clays (at all three CEC levels) were determined before and after exposure to the basal salts media. Clays prepared at 100% of their CEC were found to be expanded. The vermiculite sample yielded a d_{001} spacing of approximately 29 Å which is indicative of a paraffin complex (13). The low-charge smectite gave a 17-18 Å basal spacing which corresponds to a bilayer formation (13). HDTMA-illite samples were not analyzed by x-ray

diffraction because the inner layers are completely collapsed with K^+; all HDTMA exchanged onto the clay is externally bound and therefore does not alter x-ray diffraction patterns. Those clays prepared at levels below HDTMA-saturation showed lesser degrees of expansion. In all clays tested, the d-spacings decreased after the desorption process. The inner layers of the clays subjected to a high density of HDTMA-degrading organisms over a five-day period showed about the same collapse as those samples subjected to the desorption process. HDTMA-smectite experienced a collapse of the inner layers from 17 Å to 14 Å, the latter indicative of a mono-layer configuration of exchanged HDTMA (13). The inner layers of HDTMA-vermiculite collapsed from a paraffin (approximately 29 Å) to the mono-layer configuration (14 Å). This occurred even though only from 5 to 9% of the HDTMA was desorbed over the course of the experiment.

HDTMA-soils were prepared to estimate the relative stability of HDTMA bound to cation exchange sites of soil organic matter predominant in A horizon soils as compared to that bound to the cation exchange sites of clays predominant in B horizon soils. In desorption assays, approximately 16.1% of the HDTMA was desorbed from A horizon soil and approximately 25% from B horizon soil after nine consecutive washes in the mineral media. Approximately 75% of the Marlette A soil's CEC (11.5 meq/100 g) is due to exchange sites on organic matter. In the Marlette B horizon, 75% of the CEC (12.8 meq/100 g) is due primarily to illite and vermiculite clay minerals. When subjected to a dense culture of HDTMA-degrading bacteria, HDTMA was mineralized at the same rate and to the same extent in both soils (Figure 1.8).

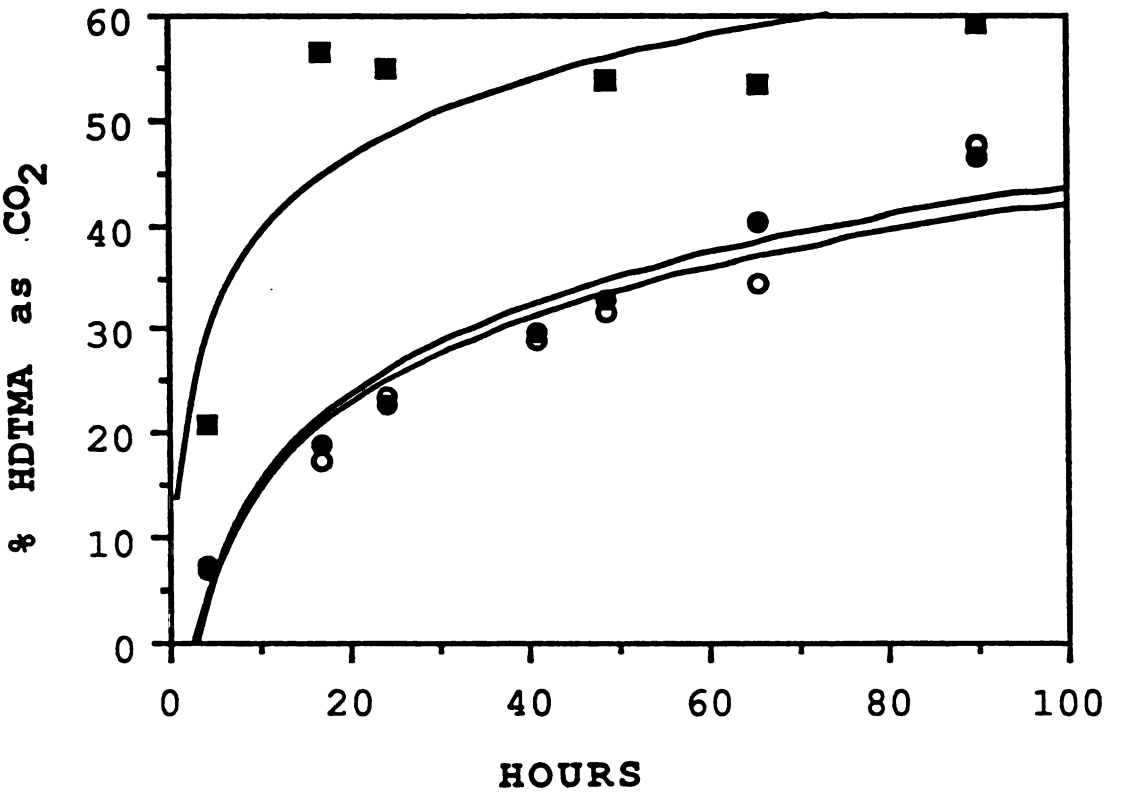

Figure 1.8. Mineralization of HDTMA bound to Marlette A and B horizons and HDTMA dissolved in water (■ Aqueous phase, ○ HDTMA-Marlette A horizon, ● HDTMA-Marlette B.)

DISCUSSION.

The labelled carbon in the ^{14}C-HDTMA used in this study was located on the terminal carbon of the hexadecyl group (away from the ammonium headgroup) to provide a conservative estimate of the biostability of organo-clays in incubations with soils. Several soil types, specifically a sandy loam soil from the A horizon (Marlette), a loam soil from the B horizon (Marlette), a loamy sand A horizon soil (Spinks), and a sandy loam B horizon soil (Spinks), as well as a petroleum-contaminated soil, were chosen to represent a range of field conditions.

Overall, the mineralization of HDTMA in soils and subsoils was low. Typically, less than 5% of the HDTMA was evolved as CO_2 during prolonged incubations of 12 to 18 months. Results of this study indicate that, while the HDTMA is generally biologically stable, the extent of mineralization is dependent on the nature of exchange sites where HDTMA is bound and on environmental conditions. Overall, the biostability of HDTMA is increased when it is added as an organo-clay complex rather than as the free cation or when introduced into subsurface (B horizon) soils versus surface (A horizon) soils.

The observed differences in the biostability of HDTMA comprising HDTMA-clays are related to the location of the exchange sites on the clay minerals. Biostability and desorption experiments have shown that HDTMA bound internally (e.g., smectite) is more stable in dilute salt solutions and against microbial attack than that which is externally bound (e.g., illite). Of the three organo-clays tested, HDTMA-smectite

is by far the most stable, both chemically and biologically due to the intercalation of HDTMA. A smectitic clay, fully exchanged with HDTMA, has a d_{001} spacing of approximately 18 Å. An average bacterial cell with a diameter of 1.5 um is approximately 750 times larger than this and therefore cannot move into the inner layers of the clay and gain direct access to the exchanged HDTMA. HDTMA-illite, on the other hand, consists primarily of mineral sheets which have no internally exchanged HDTMA due to the complete collapse of the clay interlayers when occupied by K^+ ions. Hence, in illite, all HDTMA is externally bound, apparently giving microbes direct access to the QUAT, resulting in decreased biostability. The results with HDTMA-vermiculite are more difficult to interpret. The d-spacing of the HDTMA-vermiculite was 29 Å, indicating interlayer expansion by exchanged HDTMA. Despite having HDTMA intercalated, it is mineralized to a much greater extent than observed for HDTMA-smectite. Perhaps HDTMA occupies predominantly the outermost exchange sites in vermiculite (i.e., near the edges of the clay particles) with the innermost exchange sites occupied by K^+. Because vermiculite is a limited expanding clay, the most accessible sites for HDTMA exchange are likely those near-edge sites; once HDTMA occupies these sites, it may block further access to additional exchange sites in the interior of the clay particle. The fact that HDTMA vermiculite collapses to a d_{001} spacing of 12.8 Å after desorption of only 9% of the exchanged HDTMA suggests that a small portion of the HDTMA is intercalated. This may explain why the biostability of vermiculite-bound HDTMA is somewhat more than illite-bound, yet less than that exchanged onto smectite, which is a fully expanding clay.

Desorption of bound HDTMA may also influence its overall stability. HDTMA bound to smectite was found to be the most resistant to desorption with less than 3% being removed after nine consecutive washes in a basal salts medium. HDTMA-smectite complexes were also the most biologically stable of the three HDTMA-clays tested. Organo-illite complexes experienced the greatest mineralization of bound HDTMA and the greatest desorption (between 3 and 10% desorbed after nine washes). Desorption and mineralization of HDTMA from vermiculite complexes were intermediate to those observed for smectite and illite. The ability of exchanged HDTMA to resist desorption is also related to the percent loading of the CEC with the cation. At levels nearing 100% saturation of the CEC with HDTMA, all of the HDTMA does not bind directly to the exchange sites on the mineral surfaces. Rather, some of the HDTMA is held non-ionically through non-polar interactions involving the hexadecyl groups of the exchanged HDTMA (25). Molecules held in this fashion may be more readily desorbed in the mineral salts medium. This effect is reflected in the increasing percentage desorbed with increasing CEC loading.

Long term biostability studies at 70% field capacity indicate that HDTMA added directly to surface soils is less resistant to degradation than that added in a clay-bound form. HDTMA, added as the free cation to surface soils, likely binds to both the exchange sites of soil clays and soil organic matter (SOM), the latter being more abundant than the former in the surface soils we studied. It was shown that HDTMA exchanged onto a surface soil was more readily desorbed than that

bound solely to clay minerals (16.1% for Marlette A horizon soil versus 1-7% for clays prepared at 70% of the CEC), suggesting that the avidity of binding of HDTMA to SOM sites is less than to clay sites. Hence, HDTMA added in the free form to surface soils is more readily mineralized than that added in a pre-bound state to clays, presumably because HDTMA binds primarily to SOM in surface soils. Our conclusion was supported by the similar biostabilities of HDTMA added either as the free cation or clay-bound to B horizon soils where the SOM levels are low and the cation exchange sites are derived primarily from clays.

HDTMA, free or pre-bound to clays, was more resistant to microbial degradation when introduced into subsurface (B horizon) soils than in surface (A horizon) soils. Generally, the B horizon soils contain less microbial diversity and a lower population of microbes. Also, lower concentrations of organic and inorganic growth substrates in the subsurface soils result in reduced microbial activity, and hence, greater stability of the HDTMA.

An assay was conducted to assess the mineralization of HDTMA bound to soil organic matter versus the mineral matter fraction of soils. The mineralization of HDTMA bound to Marlette A and B horizons in a culture of HDTMA degraders was followed. Despite the differences in the source of the CEC (approximately 75% of the CEC is derived from SOM in the Marlette A horizon soil), equal rates and extents of mineralization were observed. Differences in the clay mineralogy and experimental conditions employed may provide an explanation for this seeming paradox. The determination of the relative stability of SOM-bound versus clay-bound HDTMA may have been obscured in this assay in

which HDTMA clays are exposed to HDTMA-degrading organisms at high cell densities. Only smectite-bound HDTMA was resistant to attack under these vigorous conditions and the Marlette soil does not contain smectite clays.

We demonstrated that HDTMA is generally biologically stable in surface and subsurface soils; typically less than 5% of the HDTMA is mineralized during 12- to 18-month incubations. The extent of mineralization depends on the nature of the clay cation exchange sites and on the environmental conditions. Clay-bound HDTMA appeared to be more biologically stable than HDTMA bound predominantly to SOM. HDTMA-smectite complexes offer the greatest biostability, followed by HDTMA-vermiculite and -illite complexes. This decreasing biostability is also observed in desorption studies, indicating that internally-exchanged HDTMA (e.g., smectite) is the most stable because microbes are unable to gain direct access to the HDTMA. HDTMA was more susceptible to microbial attack in A horizon soils than in B horizon soils, presumably due to the higher numbers and diversity of microorganisms in the surface horizon. The observed long-term stability of HDTMA in subsoils is important because one of the potential applications of this soil modification technology is the underground injection of HDTMA to create a subsurface sorptive zone that may be useful in reducing the migration of contaminants (4). The overall stability of HDTMA-clays during long-term incubations in soils and subsoils suggests their implementability in the stabilization of contaminated soils and as additional containment barriers for hazardous waste landfills or for underground and above ground storage tanks.

REFERENCES

1. Boyd, S.A., W.F. Jaynes, and B.S. Ross. 1991. Immmobilization of organic contaminants by organo-clays: Application to soil restoration and hazardous waste containment, pp. 181-200. *In* R.A. Baker (ed.), Organic Substances and Sediments in Water, Vol. 1. Lewis Publishers, Chelsea, MI.

2. Boyd, S.A., J-F Lee, and M. Mortland. 1988. Attenuating organic contaminant mobility by soil modification. Nature. 333:345-347.

3. Boyd, S.A., Shaobai Sun, J-F Lee, and M. Mortland. 1988. Pentachlorophenol sorption by organo-clays. Clays Clay Min. 36:125-130.

4. Burris, D.R. and C.P. Antworth. 1992. In situ modification of an aquifer by a cationic surfactant to enhance retardation of organic contaminants. J. Contam. Hydrol. 10:325-337.

5. Chiou, C.T., L.J. Peter, and V.H. Freed. 1979. A physical concept of soil-water equilibria for nonionic organic compounds. Science. 206: 831-832.

6. Chiou, C.T., P.E. Porter, and D.W. Schmedding. 1983. Partition equilibria of nonionic organic compounds between soil organic matter and water. Environ. Sci. Technol. 17:227-231.

7. Chiou, C.T. and T.D. Shoup. 1985. Soil sorption of organic vapros and effects of humidity on sorption mechanism and capacity. Environ. Sci. Technol. 19:1196-1200.

8. Dean-Raymond, D. and M. Alexander. 1977. Bacterial metabolism of quaternary ammonium compounds. Appl. Environ. Microbiol. 33:1037-1041.

9. Federle, T.W. and R.M. Ventullo. 1990. Mineralization of surfactants by microbiota of submerged plant detritus. Appl. Environ. Microbiol. 56:333-339.

10. Gilbert, P. and A. Al-Taae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Letts. Appl. Microbiol. 1:101-104.

11. Grigal, D.F. 1973. Note on the hydrometer method of particle size analysis. Minnesota Forestry Research Notes, No. 245. University of Minnesota, St. Paul.

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

13. Jaynes, W.R. and S.A. Boyd. 1991. Clay mineral type and organic compound sorption by hexadecyltrimethylammonium-exchanged clays. Soil Sci. Soc. Amer. J. 55:43-48.

14. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cationic surfactants in laboratory and environmental systems. Dev. Ind. Microbiol. 24:425-434.

15. Lawrence, C.A. 1950. Surface Active Quaternary Ammonium Germicides. Academic Press, Inc., Publishers, N.Y., N.Y.

16. Lee, J-F, J.R. Crum, and S.A. Boyd. 1989. Enhanced retention of organic contaminants by soils exchanged with organic cations. Environ. Sci. Technol. 23:1365-1372.

17. Rhoades, J.D. 1982. Cation exchange capacity, pp. 149-157. *In* A.L. Page, R.H. Miller, and D.R. Keeney (ed.), Methods of soil analysis, Part 2. American Society of Agronomy, Madison, WI.

18. Ritchie, J.T. and J.R. Crum. 1989. Converting soil survey characterization data into IBSNAT crop model input, pp. 155-167. *In* J. Bouma and A.K. Brget (ed.), Land Qualities in Space and Time. Wageninger, The Netherlands.

19. Shelton, D.R. and J.M. Tiedge. 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. 48:840-848.

20. Shimp, R.J., B.S. Schwab, and R.J. Larson. 1989. Adaptation of QUAT surfactants by suspended microbial communities in a model stream. Environ. Toxicol. Chem. 8:723-730.

21. Sigoillot, J-C and M-H Nguyen. 1990. Isolation and characterization of surfactant degrading bacteria in a marine environment. FEMS Microbiol. Ecol. 73:59-68.

22. Sullivan, D.E. 1983. Biodegradation of a cationic surfactant in activated sludge. Nat. Res. 17:1145-1151.

23. Ventullo, R.M. and R.J. Larson. 1986. Adaptation of aquatic microbial communities to QUAT's. Appl. Environ. Microbiol. 51:356-361.

24. Wolin, E.A., M.J. Wolin. and R.S. Wolfe. 1963. Formation of methane by bacterial extract. J. Biol. Chem. 238:2882-2886.

25. Xu, Shihe and S.A. Boyd, unpublished data.

CHAPTER 2

BIOAVAILABILITY OF SOIL- AND CLAY-EXCHANGED HEXADECYLTRIMETHYLAMMONIUM TO TWO HDTMA-DEGRADING MICROORGANISMS

INTRODUCTION.

Hexadecyltrimethylammonium (HDTMA) is one of a number of quaternary ammonium compounds (QUATs) representing a class of commercially valuable chemicals. These surface-active compounds are used extensively in fabric softeners, disinfectants, drilling muds, hair care products, and detergents (22). They are also highly toxic to bacteria at sub-mM concentrations. However, studies with mixed (3, 10) and pure (14, 21) bacterial cultures showed that HDTMA is degradable or may serve as a sole carbon and energy source. Previous work with mixed cultures has shown that degradation of clay-bound QUAT was dependent on clay mineral type (23) or on whether sediment-bound QUAT was kept in suspension or allowed to remain settled (18).

The sorption of organic compounds to the clay and organic matter fractions of soils or sediments affects their potential for degradation by microorganisms. Many studies have shown that soil-sorbed compounds are unavailable to degradative microbes. Decreased bioavailability has been attributed to the degree of adsorption (4, 19), entrapment in soil micropores (15, 20) and soil organic matter (24), slow rates of intraparticle diffusion (5, 17), and the nature of the sorbent (23). There are, however, exceptions to this generally accepted concept of reduced bioavailability of soil- and sediment-sorbed substrates (7, 10,

18, 23) although the contributing factors are speculative. Guerin and Boyd (7) recently assessed the availability of naphthalene to two naphthalene-degrading bacteria using both a first-order kinetics model and a three-parameter, coupled degradation-desorption model they developed. They found that the availability of naphthalene was highly organism specific. A soil isolate, designated NP-Alk, was unable to utilize soil-sorbed naphthalene as mineralization rates and P_{max} values were similar to, but slightly below, values predicted if sorbed naphthalene was unavailable. For *Pseudomonas putida* (ATCC 17484), both the rates and extents of mineralization exceeded those predicted assuming only aqueous phase naphthalene was available, suggesting that the microbe was able to facilitate the desorption of sorbed naphthalene from soils. Mineralization rates which exceeded those predicted indicated that the cells of 17484 experienced naphthalene concentrations in excess of the solution phase naphthalene concentration. The organism dependent ability to access sorbed naphthalene may be related to the fact that 17484 was chemotactic toward naphthalene and attached reversibly to soil particles, while NP-Alk attached irreversibly and more extensively.

A recent study (13) has shown that HDTMA exchanged onto clays is fairly stable when incubated in agricultural and petroleum-contaminated soils (generally < 5 to 10% mineralized during incubations of one year or longer), yet clay-bound HDTMA was degraded when exposed to pure cultures of HDTMA-degrading organisms at high cell densities. Typically, 60% of the HDTMA exchanged onto illite, 35% of the HDTMA bound to vermiculite, and 10% of the HDTMA exchanged onto smectite was

recovered as CO_2 over a five-day incubation with HDTMA-degrading microbes. In this paper, we attempt to evaluate the bioavailability of HDTMA when bound to soils and clays in the presence of a pure culture of an HDTMA-degrading organism using the kinetics approach of Guerin and Boyd (7).

MATERIALS AND METHODS.

Soils and clays. Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs) soil from the B horizon (sandy loam) and Marlette (fine-loamy, mixed, mesic Glossoboric Hapludalfs) soil from both the A (sandy loam) and B (loam) horizons were collected from agricultural settings near East Lansing, Michigan. The soils were air dried, screened to pass a 2 mm sieve, and stored at room temperature. Smectite from Crook County, Wyoming (SWy) and Apache County, Arizona (SAz), illite from Silver Hill, Montana (IMt), kaolinite from Warren County, Georgia (KGa), and nontronite from Grant County, Washington (SWa) reference clays were obtained from the Source Clay Repository (Clay Minerals Society (CMS), Columbia, MO). They were wet sedimented to separate the < 2 um fraction, saturated with Mg, frozen, and freeze dried. Vermiculite from South Carolina (VSC) was obtained from the Zonolite Colloid Co. and was Na-saturated before use. Soils were characterized with respect to particle size distribution by the hydrometer method (6), organic carbon content (Dohrmann DC-190 Organic Carbon Analyzer, Rosemount Analytical, Inc., Santa Clara, CA), and cation exchange capacity (16). Soil characteristics are presented in Table 2.1.

Table 2.1. Physical and chemical properties of the soils used in this study.

Soil	Cation Exchange Capacity (meq/100 g)	% Sand	% Silt	% Clay	% Organic Carbon
Marlette A	11.2	54.9	35.7	9.4	2.2
Marlette B	12.8	51.5	31.3	17.2	0.6
Oshtemo B	3.8	89.0	5.0	6.0	0.6

The cation exchange capacities of the six reference clays have been previously determined (8) to be: 8, 24, 80, 90, 107, and 130 meq/100 g for kaolinite, illite, vermiculite, Wyoming smectite, nontronite, and Arizona smectite, respectively.

Chemicals. n-Hexadecane and HDTMA-Br (99% purity) were obtained from Sigma Chemical Co., St. Louis, MO. ^{14}C-HDTMA-Br (Moravek Biochemicals, Inc., Brea, CA, 55 mCi/mmol, 98% pure) was labelled in the C_{16} position of the hexadecyl chain, i.e., the carbon distal from the ammonium headgroup. All chemicals were used without further purification.

Bacterial cultures. Two HDTMA-degrading organisms were used in these studies. The two isolates, designated 101S and 102G, were isolated from an activated sludge sample obtained from the Lansing, Michigan municipal waste water treatment plant. An aliquot of the sludge was introduced into three flasks containing a minimal medium [high-buffer broth (HBB)(7)] along with n-hexadecane (433 uM) and HDTMA at varying concentrations (10, 100, or 1000 uM). After an initial increase in turbidity, aliquots of the growing cultures were consecutively transferred (three times) to fresh HBB containing n-

hexadecane and HDTMA. Following the transfers, an aliquot was inoculated into HBB containing HDTMA as the sole carbon source. Upon an increase in turbidity, the culture was plated onto one-half strength nutrient agar and isolated colonies were reinoculated into HBB/HDTMA. Characterization of the two isolates is in progress (14).

Determination of first-order kinetics. An experiment was designed to determine the optimum aqueous HDTMA concentration that would support first-order growth kinetics at a cell density of 10^8 mL^{-1}. 75 mL of PBS containing from 1 to 100 ug mL^{-1} HDTMA were added aseptically to 150 mL glass serum bottles. Bottles were inoculated with a washed, early stationary phase culture of 101S or 102G at a final cell density of 10^8 ml^{-1} (standardized by A_{600} measurements, Spectronic 88 spectrophotometer), crimp-capped with Teflon-lined septa, and incubated at 25 °C on a rotary shaker (150 rpm). At predetermined intervals, 1 mL of the aqueous phase and 1 mL of the gaseous phase in the bottle were removed by syringe, transferred to a tube containing 1 mL 2 N HCl, and the evolved $^{14}CO_2$ was trapped on KOH-saturated filter paper suspended from the tube stopper in a plastic cup. Tubes sat overnight to allow quantitative recovery of $^{14}CO_2$. Filter papers were transferred to glass scintillation vials containing 7.5 mL of Safety-Solve scintillation counting cocktail for aqueous solutions (Research Products International Corp., Mt. Prospect, IL) along with 3 mL of 95% ethanol used to rinse the plastic cup. The ^{14}C activity was measured by liquid scintillation counting (LSC) on a Packard 1500 TriCarb Liquid Scintillation Analyzer (Packard Instrument Co., Downer's Grove, IL) using external standard quench correction.

^{14}C-HDTMA bioavailability assays. The mineralization kinetics approach for assessing sorbed compound bioavailability described by Guerin and Boyd (7) was used in this study. Sterile soils or clays pre-equilibrated with solutions of HDTMA (in PBS) at concentrations within the first-order domain of the organism-specific Michaelis-Menten kinetics were inoculated with non-growing cells of HDTMA-induced bacteria. Due to the range of CECs for the sorbent materials used in this study, only a few milligrams or less of the sorbent was required to provide cation exchange capacity equivalent to the HDTMA contained in 75 mL of a 1 ug mL^{-1} solution. Due to the inability to accurately weigh such small quantities, aliquots of sterile suspensions (< 1 mg mL^{-1}) of the illite, Wyoming smectite, and vermiculite clays were volumetrically pipetted into 150 mL serum bottles. Because the CECs of the kaolinite clay and the soils were sufficiently low, greater masses of these sorbents were used, and they could be accurately weighed and added directly to the serum bottles. Sorbents were equilibrated overnight with solutions containing 1 ug mL^{-1} HDTMA including sufficient ^{14}C-HDTMA to attain initial total activities of approximately 5,000 dpm mL^{-1}. Sorbent-free controls contained solutions of HDTMA varying in concentration from 0.1 to 1 ug mL^{-1}. Early stationary phase cells collected by centrifugation (6,500 x *g*, 10 minutes) and washed and resuspended in PBS served as inocula added to achieve a final cell density of 10^8 mL^{-1}. Serum bottles were sealed with Teflon-lined septa, crimp-capped and incubated at 25 °C on a rotary shaker. At precisely timed intervals, 1 mL of the aqueous phase and 1 mL of the gaseous phase

in the bottle were removed by syringe and transferred to tubes as previously described for determination of $^{14}CO_2$ produced.

Bioavailability of HDTMA at various CEC:HDTMA. HDTMA was added to clay suspensions in which the amount of clay varied to provide cation exchange capacity equal to 0.5 to 50 times the equivalents of HDTMA. A sterile suspension of Arizona smectite (16 mg mL^{-1}) was prepared in PBS and allowed to equilibrate overnight. The clay was collected by centrifugation (8,000 x *g*, 15 minutes), and resuspended in PBS three consecutive times. Aliquots of this suspension (containing from 0.114 mg to 11.4 mg of Arizona-smectite) were equilibrated overnight in 150 mL serum bottles containing a total volume of 75 mL PBS with 1 ug mL^{-1} HDTMA, including sufficient ^{14}C-HDTMA to achieve total initial activities of 5,000 dpm mL^{-1}. The clay provided 0.5, 1, 2, 5, 10, 20, and 50 times the cation exchange capacity relative to the number of equivalents of HDTMA in each bottle. Early stationary phase 102G cells, collected by centrifugation, washed and resuspended to a final density of 10^{10} cells, served as inocula which were added to the bottles to achieve a final cell density of 10^8 mL^{-1}. At precisely timed intervals over a five day period, subsamples were removed and analyzed as previously described to determine $^{14}CO_2$ production.

Effect of Charge Location on HDTMA bioavailability. An experiment was designed to assess the effect of tetrahedral versus octahedral charge on the bioavailability of smectite-bound HDTMA. Sterile suspensions (16 mg mL^{-1}) of Mg-saturated nontronite (charge primarily in tetrahedral layer) and Arizona smectite (charge primarily in octahedral layer) in 10 mM NaCl were prepared by equilibrating the

clay overnight with the subsequent collection by centrifugation and resuspension in the NaCl solution five times to saturate the cation exchange sites with Na^+. Aliquots of these suspensions (containing cation exchange capacity equal to 0.1, 1, 10, and 100 times the equivalents of HDTMA) were added to serum bottles containing 25 mL of a 1 ug mL^{-1} HDTMA solution (5,000 dpm mL^{-1}) in distilled water and equilibrated overnight to allow HDTMA exchange. Following equilibration, volumes were brought to 75 mL with the addition of PBS. Mineralization was initiated upon the addition of washed, early stationary phase cells of isolate 102G resuspended in PBS and added to achieve a final cell density of 10^8 mL^{-1}. Sampling and analysis procedures have been described above.

Kinetic model to assess bioavailability. Mineralization data (percentage, P, of the total initial activity converted to $^{14}CO_2$ as a function of time, t) were fitted to a first order production equation:

$$P = P_{max} (1 - e^{-kt}) \quad (1)$$

to derive estimates of P_{max} (the maximum percentage mineralized), and k (the first-order mineralization rate constant) by non-linear regression analysis (SYSTAT). Initial mineralization rates (ug mL^{-1} min^{-1}) were obtained by normalizing k to P_{max} and the initial HDTMA concentration. Specific mineralization rates (ug $cell^{-1}$ min^{-1}) were calculated for inter-experiment comparisons by dividing the initial mineralization rates by the experimental cell densities determined by plate counts of the inoculum at the start of the experiments.

Assessment of bioavailability. At concentrations below the half-saturation constant, K_m, for non-growing cells, mineralization rates are

directly proportional to substrate concentration (1, 9). If sorbed HDTMA is unavailable to bacteria, increases in the sorbent to solution ratio will concomitantly decrease the equilibrium aqueous phase concentration of HDTMA and result in decreases in initial mineralization rates (7). For sorbent-free systems, a plot of HDTMA concentration versus the initial mineralization rate will result in a straight line which theoretically passes through the origin. In HDTMA-sorbent systems, plots of initial mineralization rates versus the equilibrium aqueous phase concentrations should fall on the sorbent-free control line *if sorbed HDTMA is unavailable* (Figure 2.1). Data points which fall above the theoretical line indicate that additional HDTMA besides that in the aqueous phase is immediately available (i.e., the HDTMA degraders experience HDTMA concentrations in excess of the aqueous phase HDTMA concentration).

P_{max} can also be used to assess the availability of a bound substrate. If mineralization is limited by sorption and if desorption rates are slow relative to biodegradation rates, P_{max} values for HDMTA-sorbent systems should fall on a line connecting the soil-free P_{max} value with the origin in a plot of P_{max} versus the equilibrium aqueous HDTMA concentration (Figure 2.1).

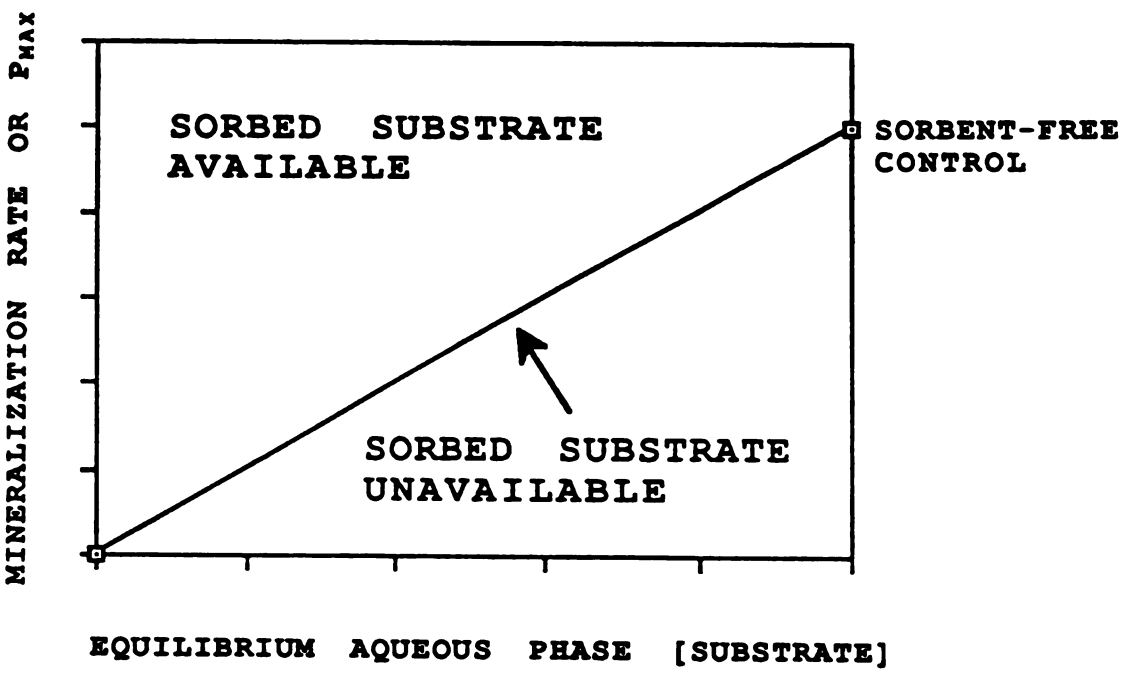

Figure 2.1. Model predicting the bioavailability of sorbed substrates based on mineralization rates or P_{max} values obtained from sorbent-free controls at various equilibrium aqueous phase concentrations of the substrate.

RESULTS.

To determine the relationship between mineralization rates and HDTMA concentrations, mineralization time courses were conducted for both organisms at HDTMA concentrations of 0.1 to 1.0 ug mL^{-1} and initial cell densities of 10^8 mL^{-1}. Higher HDTMA concentrations resulted in unfavorable mineralization kinetics with considerable lag periods preceding degradation as shown in Figure 2.2. Initial mineralization rates were calculated from data at the lower concentrations (0.1 to 1.0 ug mL^{-1}). When expressed in a double reciprocal Lineweaver-Burk plot of HDTMA concentration versus initial mineralization rates, K_m and V_{max} parameters were poorly defined for both organisms. The regression line passed through the origin which made it impossible to calculate the desired parameters (Figure 2.3). Nevertheless, this experiment established the linear relationship between initial mineralization rate and the aqueous HDTMA concentration over this concentration range.

Bioavailability of sorbed HDTMA was assessed in pre-equilibrated (24 hours) HDTMA-clay or -soil systems inoculated with known HDTMA-degrading bacteria at a cell density of approximately 10^8 mL^{-1}. A series of sorbent-free mineralization controls were set up to obtain the theoretical line for the rates of mineralization at varying equilibrium solution phase concentrations. Figure 2.4 shows the mineralization time courses for clay-free controls and HDTMA-illite suspensions inoculated with HDTMA-induced cells of isolate 102G. Sorption-dependent decreases in both the apparent rates and extents of HDTMA mineralization were evident. Partition controls, which mimicked conditions in the

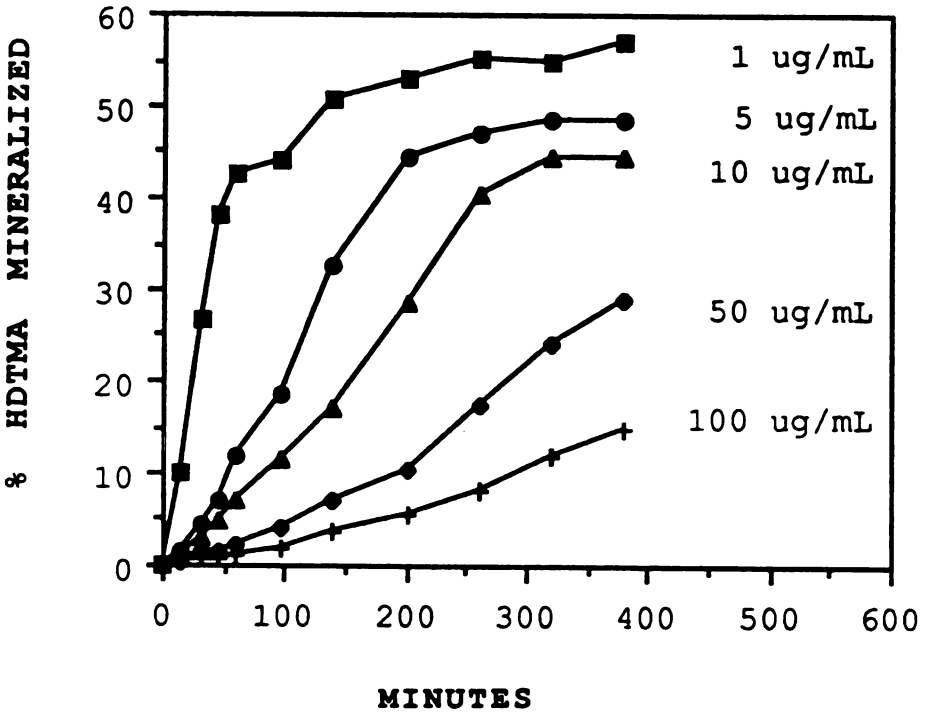

Figure 2.2. Mineralization time courses for varying HDTMA concentrations with 10^8 cells mL^{-1} (102G).

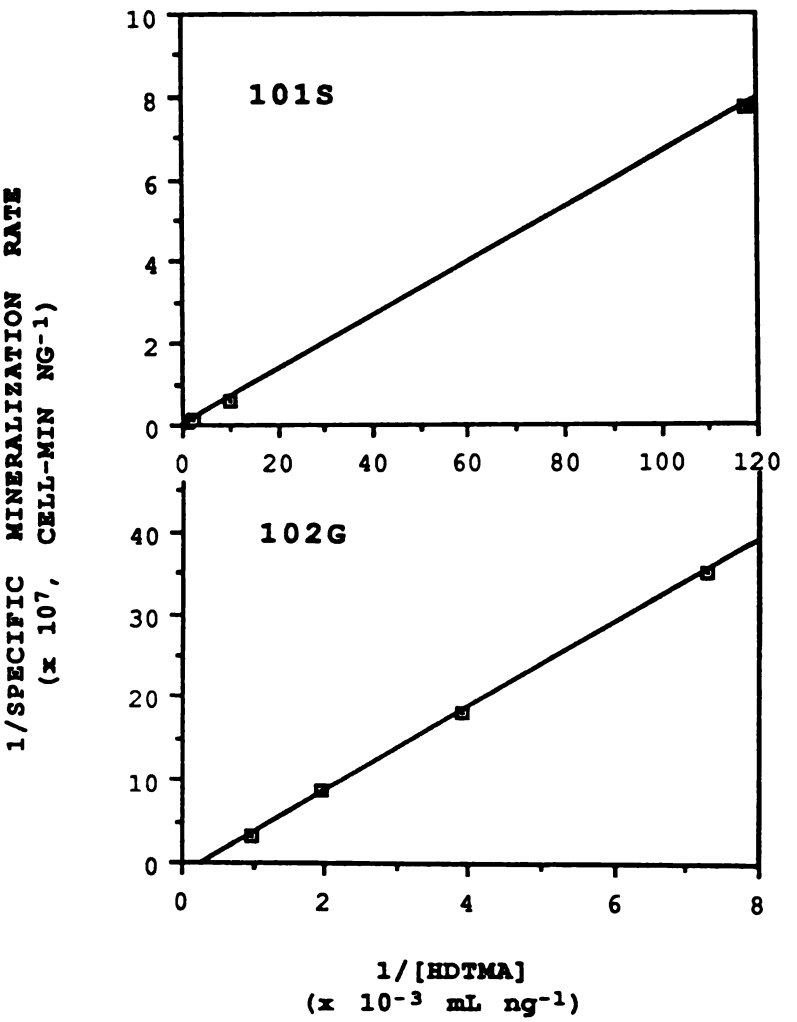

Figure 2.3. Lineweaver-Burk plots of the linear relationship between the rates of HDTMA mineralization and [HDTMA] for organisms 101S and 102G.

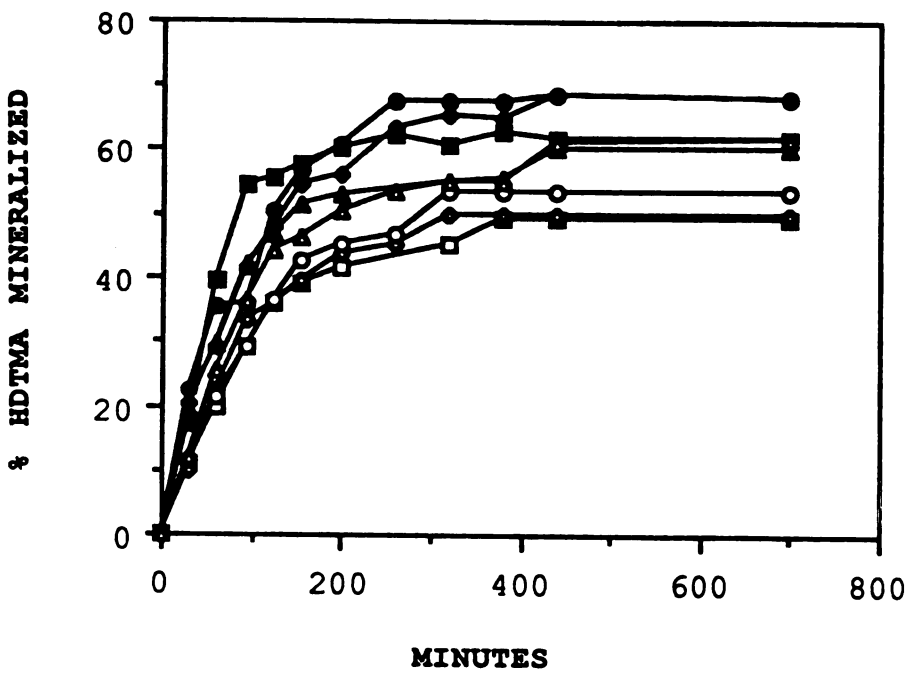

Figure 2.4. Representative mineralization curves for bioavailability assays showing mineralization time courses for sorbent-free (solid symbols) and illite-containing systems (open-symbols) inoculated with 102G. Equilibrium aqueous phase concentrations of HDTMA in sorbent-containing systems were approximately equivalent to those in sorbent-free systems.

mineralization assays, were set up to determine the actual equilibrium aqueous phase concentrations of HDTMA in the presence of the sorbents, but provided no conclusive data. Centrifugation did not successfully pellet the small masses of clay and soil particles present in the partition controls. HDTMA sorption to filter paper confounded filtration techniques. As a result, the aqueous phase HDTMA concentration at equilibrium in soil or clay-containing systems was calculated using the CEC of the sorbent and assuming stoichiometric exchange of the HDTMA. Adsorption isotherms of HDTMA by soils (11) and clay (8) have shown that HDTMA is very strongly adsorbed when added in an amount $\leq$ the CEC of the soil or clay. Mineralization data obtained in the assays (Figure 2.4) were fit to equation (1) to derive estimates of k and P_{max}. Initial mineralization rates were then calculated and plotted as a function of the equilibrium aqueous phase HDTMA concentration. The overall specific rates of mineralization observed in the clay- and soil-containing systems were relatively constant at different levels of soil or clay, i.e., were independent of calculated HDTMA aqueous phase concentrations. Thus, as the sorbent to solution ratio increased, mineralization rates fell well above the line. At high sorbent to solution ratios, the initial HDTMA mineralization rates in soil- or clay-slurry systems and for both organisms substantially exceeded rates predicted from sorbent-free bottles assuming sorbed HDTMA is unavailable. These data indicated that some sorbed HDTMA was bioavailable (Figures 2.5 and 2.6). In general, there was better agreement between measured and predicted rates at lower soil to solution ratios (higher equilibrium aqueous phase [HDTMA]) which indicated that

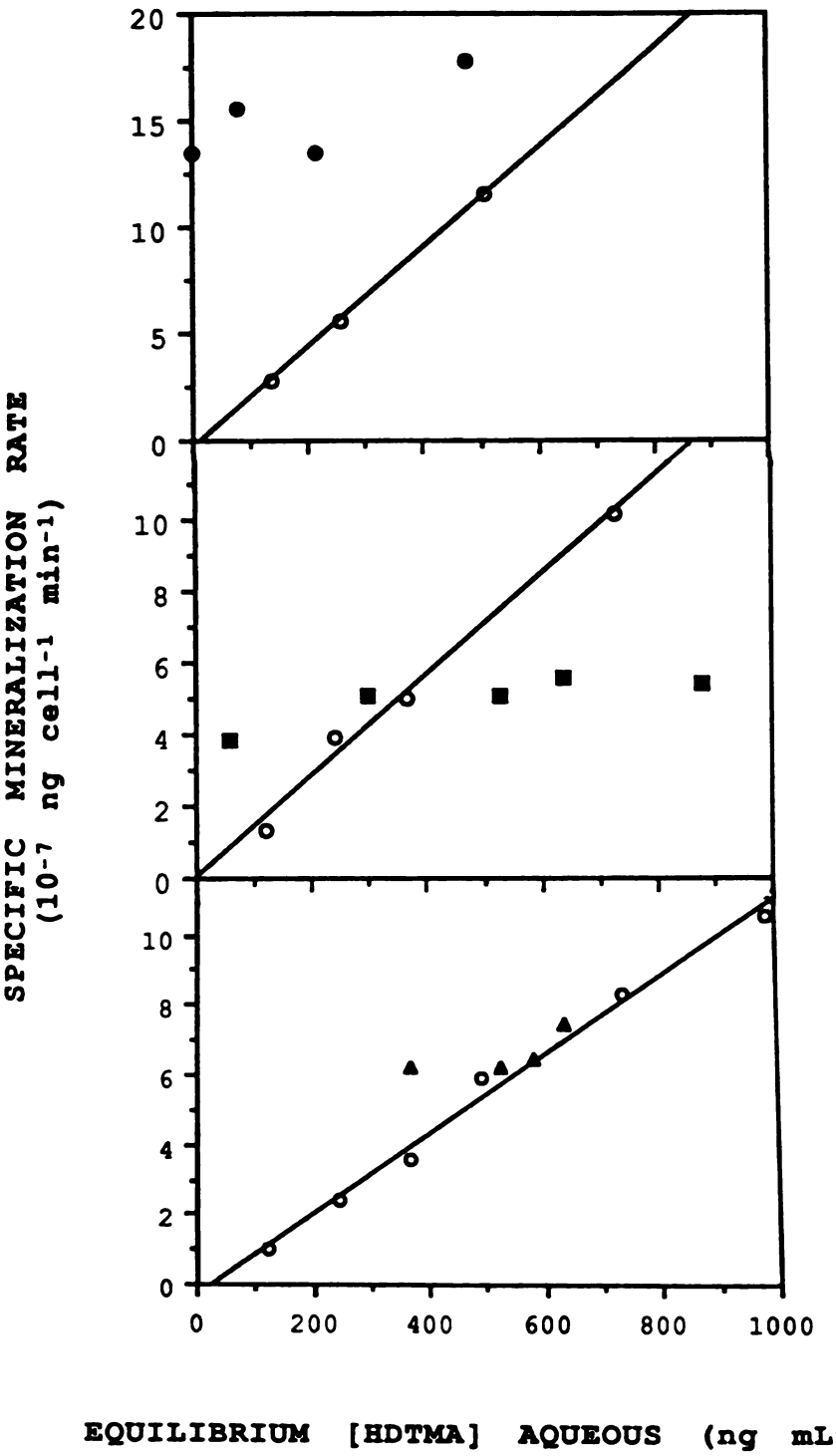

Figure 2.5. Plots of mineralization rates versus the equilibrium aqueous phase HDTMA concentrations for illite (●) and Wyoming smectite (■) clays, and Oshtemo B horizon soil (▲) as compared to sorbent-free controls (○) in systems inoculated with 102G (10^8 cells mL^{-1}).

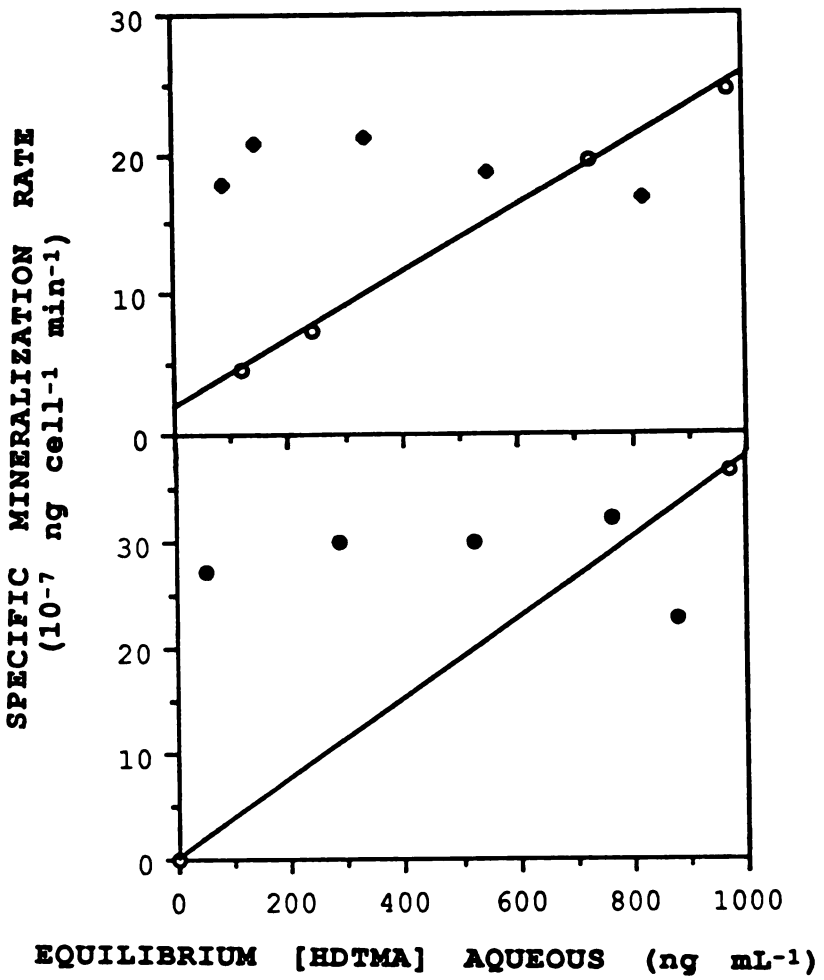

Figure 2.6. Plots of mineralization rates versus the equilibrium aqueous phase HDTMA concentrations for kaolinite (◆) and vermiculite (●) clays as compared to clay-free controls (o) in systems inoculated with 10^8 cells mL^{-1} isolate 102G.

sorbed HDTMA was unavailable. Additionally, at some of the lower sorbent to solution ratios, data points for sorbent-containing systems fell below the theoretical line indicating that the cells experienced HDTMA concentrations less than that calculated to be present in solution.

A comparison of the P_{max} values obtained in the sorbent-containing and sorbent-free systems showed that the extent of mineralization was reduced due to sorption (Figure 2.7). Typically, 70 to 80% of the added radioactivity was recovered as $^{14}CO_2$ in sorbent-free systems. Calculated P_{max} values for sorbent-containing systems were 50 to 60%, but were also independent of equilibrium aqueous HDTMA concentrations. This suggests that a fixed percentage of the added HDTMA was always available regardless of the amount of sorbent in the systems at levels equal to or below that needed to exchange all of the HDTMA. The near zero slopes of the asymptotes of the mineralization curves suggest that desorption was slow compared to biodegradation rates and had little, if any, effect on the mineralization kinetics over the time course of the experiment. This was indicated in the mineralization time courses which show no additional HDTMA mineralization from 6 to 12 hours (Figure 2.4). Desorption of bound HDTMA during this time would lead to its subsequent mineralization to $^{14}CO_2$. This was not the case as shown in Figures 2.4 and 2.8.

Due to the results which indicated that a fixed portion of HDTMA was available irrespective of the amount of sorbent added, an experiment was designed to assess the effects of increasing the CEC:HDTMA ratio from less than 1:1 to many times greater (50:1). This allowed for a

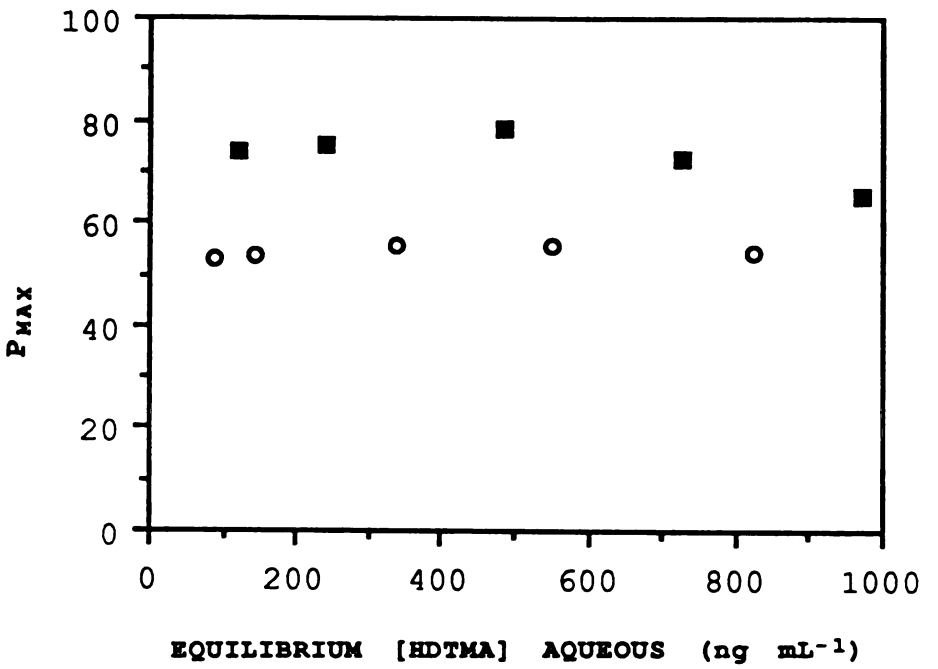

Figure 2.7. Plot of P_{max} versus the equilibrium aqueous phase HDTMA concentration for sorbent-free (■) and kaolinite-containing (○) systems inoculated with 102G cells at a density of 10^8 mL^{-1}.

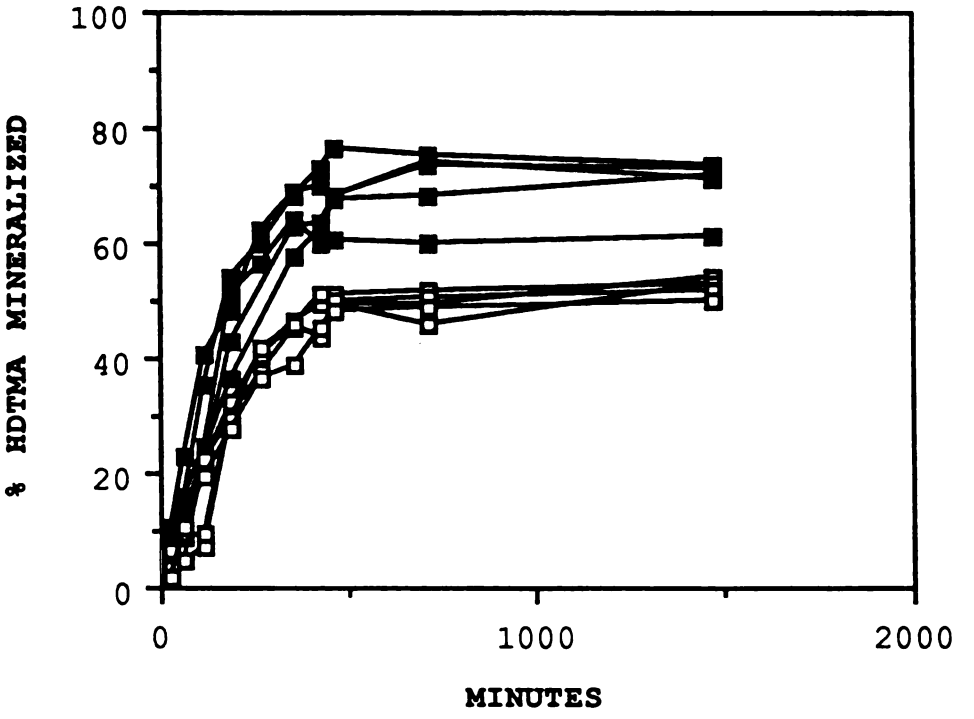

Figure 2.8. Mineralization time courses for sorbent-free (■) and kaolinite-containing (□) systems inoculated with 10^8 cells mL^{-1} 102G. Equilibrium aqueous phase HDTMA concentrations for both systems ranged from 120 to 1000 ng L^{-1}.

comparison of the availability of HDTMA in sorbent-containing systems with some unexchanged HDTMA remaining in solution and in systems where all of the HDTMA is expected to be exchanged onto the clay surface due to the excess cation exchange sites available. Figure 2.9 presents the mineralization time courses for the described study with Arizona smectite at CEC:HDTMA ratios ranging from 0.5:1 to 50:1. The rates of HDTMA mineralization were reduced in the smectite-containing systems as compared to the clay-free control, while the extents of mineralization varied from greater than the control at the CEC:HDTMA ratios $\leq$ 1:1 to less than the control at CEC:HDTMA ratios > 1:1. At a CEC:HDTMA of 50:1 all of the HDTMA in the system should be ionically bound, yet approximately 50% of the ^{14}C-HDTMA was mineralized to $^{14}CO_2$ over the course of the assay. The P_{max} values for the sorbent-containing flasks show that the extent of mineralization is relatively insensitive to the CEC:HDTMA ratio.

HDTMA exchange onto Na-smectite is more favorable in low ionic strength solutions. High salt concentrations, such as those present in PBS, could cause the clay to flocculate, reducing accessibility to the clay interlayers. Conversely, HDTMA access to the inner layer exchange sites of the smectite would be maximized in low ionic strength solutions which promote clay swelling, coupled with the reduced competition from other exchangeable cations. The experimental data clearly show that HDTMA exchange of Na-smectite in a solution of low ionic strength ($I < 0.004$) (Figure 2.10), decreased the availability of HDTMA compared to HDTMA exchange in PBS ($I \cong 0.168$) (Figure 2.9). For the HDTMA-Arizona smectite systems prepared at low ionic strength, only the 0.1:1

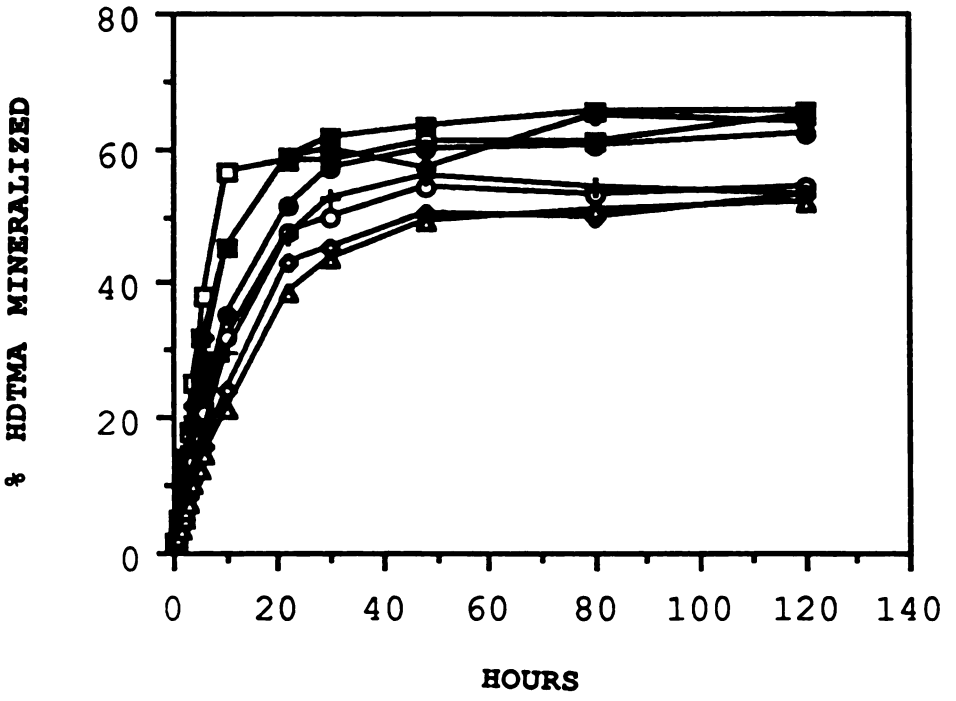

Figure 2.9. Mineralization time courses for sorbent-free (□) and Arizona smectite containing systems at various CEC:HDTMA (● 0.5:1, ■ 1:1, ● 2:1, ○ 5:1, ✚ 10:1, ◆ 20:1, ▲ 50:1.) exchanged in PBS and inoculated with 102G.

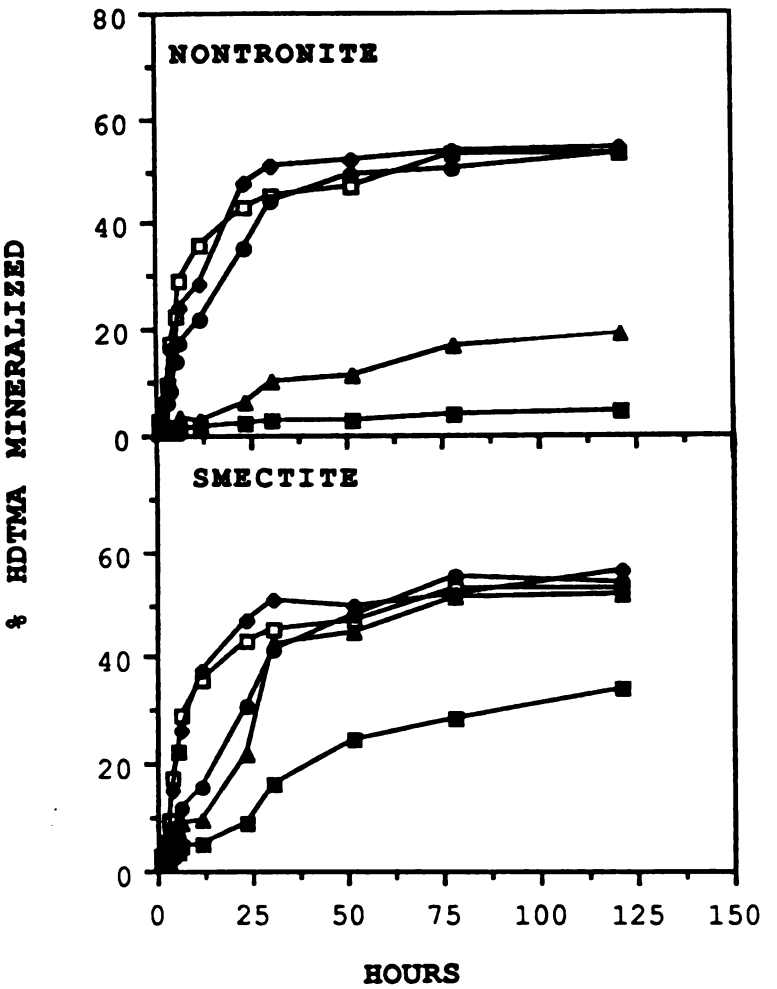

Figure 2.10. Mineralization curves for sorbent-free (□) and nontronite and Arizona smectite-containing systems at various CEC:HDTMA (● 0.1:1, ● 1:1, ▲ 10:1, ■ 100:1) with HDTMA exchange occurring in dilute NaCl solutions.

ratio of CEC:HDTMA resulted in mineralization rates equivalent to those of the control bottles. CEC:HDTMA ratios of 1:1, 10:1, and 100:1 showed increasing lag periods prior to increased rates of mineralization. However, despite these periods of slower mineralization rates, P_{max} values for all systems except the 100:1 ratio eventually equalled or exceeded the extent observed for the clay-free control.

Plots of the CEC:HDTMA ratios versus mineralization rates and P_{max} (Figure 2.11) show that both the rates and extents of HDTMA mineralization were higher for HDTMA-smectite prepared in PBS as compared to those prepared in dilute NaCl. A comparison of P_{max} values at a CEC:HDTMA of 50:1 for both smectite-containing systems (PBS vs. NaCl) clearly shows this trend. For HDTMA-smectite prepared in PBS, P_{max} equalled approximately 53%; the extent was reduced to 46% for HDTMA-smectite exchanged in dilute NaCl.

The location of the layer charge also influences the bioavailability of sorbed HDTMA. The charge on nontronite is located in the tetrahedral layer (surface-localized), while that of Arizona smectite is located internally (octahedral layer). For nontronite, CEC:HDTMA ratios of 0.1:1 and 1:1 resulted in mineralization rates and P_{max} values similar to those of the clay-free controls, while ratios of 10:1 and 100:1 showed considerably slower rates and much lower extents of mineralization (Figure 2.10). Figure 2.11 shows that the rate and extent of mineralization of HDTMA from nontronite are much less than for HDTMA from Arizona smectite. In fact, at a CEC:HDTMA of 100:1, only 5% of the HDTMA was mineralized to CO_2 as compared to approximately 40% for the respective smectite-containing system. In both the nontronite and

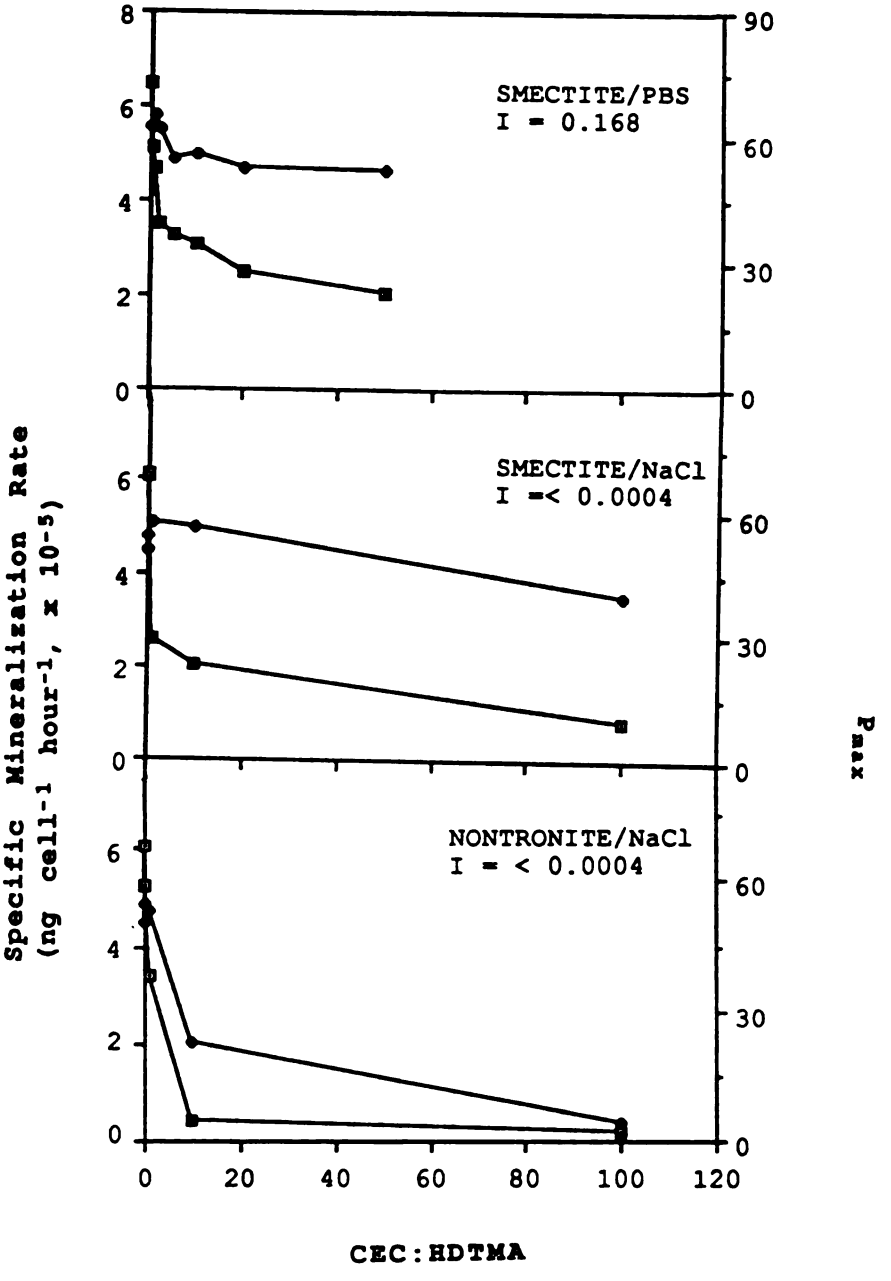

Figure 2.11. Effect of exchange site location and ionic strength of medium supporting HDTMA exchange on the mineralization rates (⊡) and P_{max} values (◆) at various CEC:HDTMA.

smectite-containing systems where HDTMA exchange occurred in dilute NaCl, there appears to be a biphasic mineralization curve showing a period of rapid mineralization with a rate somewhat less than the initial rate (Figure 2.10). This biphasic nature was not observed for the Arizona smectite systems with HDTMA exchange occurring in PBS (Figure 2.9).

To determine whether organic- and mineral-matter bound cations are equally unavailable, the mineralization kinetics of HDTMA bound to Marlette A (cation exchange sites primarily organic) versus the Marlette B (cation exchange sites primarily mineral) horizon soils were measured. Figure 2.12 shows that the mineral and soil organic matter bound cations are equally available to microbial degraders with rate data for both soils falling above the theoretical line. Marlette A soil has a CEC of 11.6 meq/100 grams, 75% of which is due to organic matter exchange sites. Soil clays are responsible for approximately 75% of the corresponding B horizon soil's CEC of 12.8 meq/100 grams.

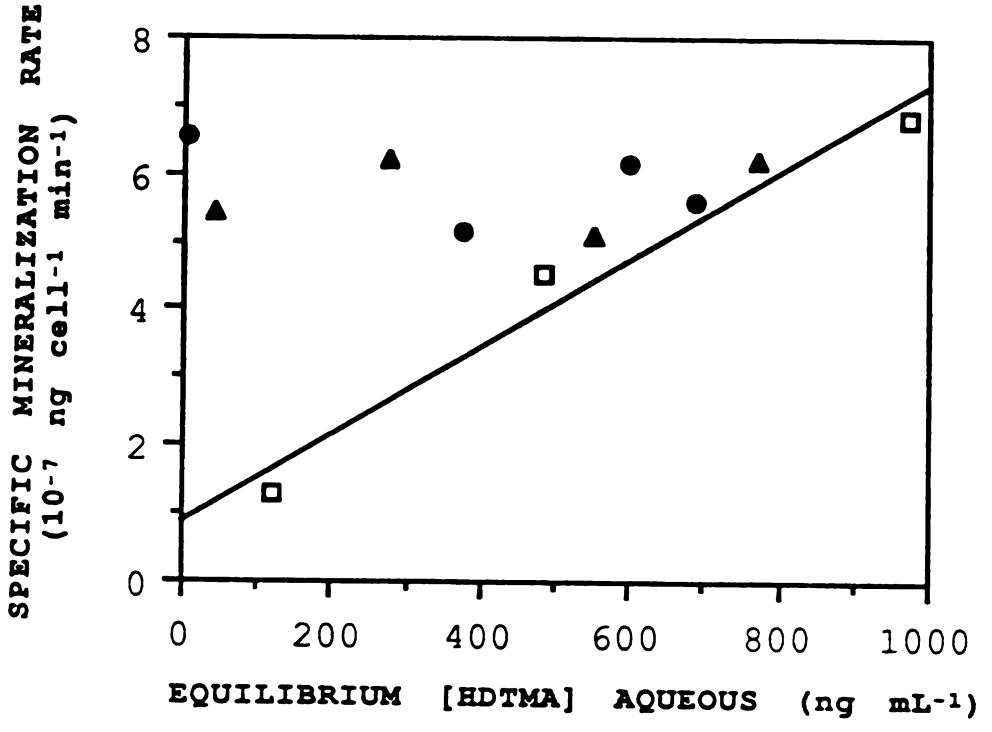

Figure 2.12. Plots of mineralization rates versus the equilibrium aqueous phase HDTMA concentrations for Marlette A horizon soil (▲) and Marlette B horizon soil (●) as compared to soil-free controls (□) (10^8 cells mL^{-1} isolate 102G).

DISCUSSION.

If adsorbed HDTMA is biologically unavailable, the mineralization rates in the presence of sorbents will be a linear function of the aqueous phase substrate concentration. In an earlier report, sediment-bound dodecyltrimethylammonium (DODMA) was shown to be unavailable to sediment microbes as evidenced by mineralization rates which were a function of the aqueous phase DODMA concentration rather than of the total mass of DODMA present in the system (10). The bioavailability assay data presented here clearly show that HDTMA sorption acted to decrease the extent of mineralization of HDTMA relative to sorbent-free systems, yet HDTMA in soil- or clay-containing systems was frequently mineralized at a greater rate than predicted from the calculated aqueous phase HDTMA concentrations assuming that sorbed HDTMA is unavailable. Bacteria in sorbent-containing systems experienced a higher concentration of HDTMA than that calculated to be present in solution and therefore, the rates of mineralization in these systems often fell well above the theoretical rates predicted from sorbent-free systems. Essentially constant rates were observed even as the aqueous phase concentration was presumably lowered due to sorption. These results suggest that a constant mass of bound HDTMA was immediately available to microbial degraders. Studies have shown that montmorillonite-containing systems eliminate the availability of diquat (23) and benzylamine (12) for microbial degradation, while others have shown that clay (23) and sediment-sorbed (10) QUATs are bioavailable.

With increasing sorbent concentrations, and hence decreasing equilibrium aqueous phase HDTMA concentrations, HDTMA mineralization rates and P_{max} values should also decrease if sorbed HDTMA is not available to microbial degraders. Measured mineralization rates at higher solids to solution ratios in our assays were consistently much higher than rates predicted when assuming only aqueous phase HDTMA was available. Also in these experiments, constant P_{max} values at varying sorbent concentrations (Figure 2.7) indicated that a constant percentage of the total mass of HDTMA in the system (bound and solution phase) was always available. There are two possible explanations for this observation: 1) sorbed HDTMA desorbed and became available or 2) a portion of the sorbed HDTMA was bioavailable. No significant desorption was observed over the time course of these assays as mineralization of HDTMA reached a plateau with no subsequent mineralization as the assay progressed (Figure 2.8). Hence, the constant P_{max} values in sorbent-containing systems suggest that some sorbed HDTMA was available for microbial degradation. Clays and soils generally have high affinities for QUATs containing large organophilic moieties, such as HDTMA, resulting in nearly stoichiometric exchange of native inorganic cations and high selectivity coefficients (2, 7, 11). Recently, more detailed studies of HDTMA exchange reactions on subsoils have shown that at levels nearing 100% saturation of the CEC with HDTMA, all of the HDTMA does not bind directly to exchange sites (25). Rather, some of the HDTMA is held non-ionically through non-polar interactions among the hexadecyl groups of the exchanged HDTMA (hereafter referred to as tail-tail interactions). Cations held through these weaker interactions may

be more accessible to microbial degraders than HDTMA held by cation exchange. This may explain the availability of a portion of the HDTMA, however, some unrecognized subtleties in the HDTMA exchange reactions may have resulted in a constant percentage of the HDTMA becoming bound at different sorbent levels, manifesting constant mineralization rates and P_{max} values.

The availability of sorbed HDTMA was altered by conditions during HDTMA-exchange. Na-saturation of smectite clays, and the addition of HDTMA in a medium of low ionic strength, favors inner layer expansion and intercalation of the HDTMA which decreases the overall bioavailability of the sorbed HDTMA. In HDTMA-smectite systems exchanged in dilute NaCl, initially high mineralization rates were followed by a plateau and then a continuation of mineralization, but at a slower rate, a trend not observed in the systems exchanged in PBS. This suggests that when HDTMA is exchanged in a low ionic strength medium, it sorbs to more remote (less accessible) sites. In these systems, HDTMA exchanged onto the near-edge sites may be removed and mineralized more readily, followed by a lag and then mineralization of less accessible HDTMA. HDTMA-clays and -soils (Figures 2.5, 2.6, and 2.12) prepared in high ionic strength PBS may have been subject to non-ideal exchange behavior (i.e., incomplete or weak bonding of HDTMA). As a result, a percentage of the HDTMA is available, while only a small portion remains permanently bound.

The location of the layer charge in clays was a determinant of the bioavailability of sorbed HDTMA. Greater bioavailability was observed for HDTMA exchanged onto Arizona-smectite clay compared to the

nontronite-exchanged HDTMA. Because the charge is surface-localized in nontronite, perhaps this clay binds HDTMA more avidly than in Arizona smectite where the charge is located primarily in the octahedral layer which is more distant from the clay surface.

The observed bioavailability of soil- or clay-bound HDTMA can be decreased by increasing the CEC:HDTMA ratio. In HDTMA-nontronite systems, P_{max} is decreased by approximately 2.5 times as compared to sorbent-free systems over a five-day period for a CEC:HDTMA of 10:1. At a ratio of 100:1, the P_{max} reaches only 10% of the respective sorbent-free system. Increasing the CEC:HDTMA ratios to many times greater than 1:1 should increase the percentage of the HDTMA intercalated in smectite and HDTMA held solely by cation exchange mechanisms rather than non-polar (tail-tail) interactions, thereby decreasing the bioavailability of the HDTMA. HDTMA concentrations in excess of the sorbent's CEC will result in significant tail-tail interactions and HDTMA that may be bioavailable.

The results presented here regarding the bioavailability of clay- and soil-exchanged HDTMA have raised some interesting issues which need to be more fully addressed. Despite a reduction in the extent of mineralization of HDTMA due to sorption, the rates of mineralization usually exceeded the predicted rates for sorbent-free systems assuming sorbed substrate is unavailable. Also, essentially constant mineralization rates were observed in sorbent-containing systems even as the aqueous phase concentrations of HDTMA were presumably lowered due to HDTMA sorption. Constant P_{max} values at various sorbent to solution ratios suggest that a fixed percentage of the HDTMA is immediately and

ultimately available to microbial degraders. Environmental conditions during HDTMA exchange also influenced bioavailability of sorbed HDTMA. Na-saturation of the clays and the addition of HDTMA in a medium of low ionic strength were shown to decrease HDTMA bioavailability. HDTMA exchange onto nontronite clay with a surface-localized charge in the tetrahedral layer was less available than HDTMA bound to smectite clay where the charge is located primarily in the octahedral layer (away from the clay surface). Increasing the CEC:HDTMA ratios decreased the availability of HDTMA to microbial degraders, likely due to an increased percentage of the HDTMA held by cation exchange mechanisms rather than by the weaker, non-polar (tail-tail) interactions. The mineralization kinetics approach we used to assess the bioavailability of sorbed HDTMA, has shown that HDTMA exchanged onto a variety of clays and soils is generally available for degradation by pure cultures of HDTMA degraders at high cell densities. However, in order to fully explain the results presented, a more complete understanding of the state of sorbed HDTMA needs to be obtained. The total amount of HDTMA sorbed in each sorbent-clay system, the location of the bound HDTMA, the sorptive mechanisms involved, and the methods by which the bacterial degraders access sorbed HDTMA are all important considerations regarding this issue. Knowledge of these parameters will provide insight on the availability of sorbed HDTMA and may clarify the issues discussed here.

REFERENCES

1. Alexander, M. and K.M. Scow. 1989. Kinetics of biodegradation in soil, p. 243-269. *In* B.L. Sawhney and K. Brown, (ed.), Reactions and Movement of Organic Chemicals in Soil. Soil Science Society of America special publication, No. 22, Soil Science Society of America, Madison, WI.

2. Boyd, S.A., J-F Lee, and M.M. Mortland. 1988. Attenuating organic contaminant mobility by soil modification. Nature. 333:345-347.

3. Dean-Raymond, D. and M. Alexander. 1977. Bacterial metabolism of quaternary ammonium compounds. Appl. Environ. Microbiol. 33:1037-1041.

4. Gordon, A.S. and F.J. Millero. 1985. Adsorption mediated decrease in the biodegradation rate of organic compounds. Microbiol. Ecol. 11:289-295.

5. Greer, L.E. and D.R. Shelton. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-dichlorophenoxyacetic acid degradation in soil. Appl. Environ. Microbiol. 58:1459-1465.

6. Grigal, D.F. 1973. Note on the hydrometer method of particle size analysis. Minnesota Forestry Research Notes, No. 245, University of Minnesota, St. Paul.

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

8. Jaynes, W.F. and S.A. Boyd. 1991. Clay mineral type and organic compound sorption by hexadecyltrimethylammonium-exchanged clays. Soil Sci. Soc. Amer. J. 55:43-48.

9. Larson, R.J. 1980. Environmental extrapolation of biotransformation data. Role of biodegradation kinetics in predicting environmental data. p. 67-86. *In* A.W. Maki, K.L. Dickinson, and J.Cairns, Jr. (ed.), Biotransformation and Fate of Chemicals in the Aquatic Environment. American Society for Microbiology, Washington, D.C.

10. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cationic surfactants in laboratory and environmental systems. Dev. Ind. Microbiol. 24:425-434.

11. Lee, J-F, J.R. Crum, and S.A. Boyd. 1989. Enhanced retention of organic contaminants by soils exchanged with organic cations. Environ. Sci. Technol. 23:1365-1372.

12. Miller, M.E. and M. Alexander. 1991. Kinetics of bacterial degradation of benzylamine in a montmorillonite suspension. Environ. Sci. Technol. 25:240-245.

13. Mueller, S.A., W.F. Guerin, and S.A. Boyd, Chapter 1, unpublished data.

14. Mueller, S.A., W.F. Guerin, and S.A. Boyd, Chapter 3, unpublished data.

15. 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. 49:582-587.

16. Rhoades, J.D. 1982. Cation exchange capacity, pp. 149-157, *In* A.L. Page, R.H. Miller, and D.R. Keeney (ed.) Methods of Soil Analysis, Part 2, American Society of Agronomy, Madison, Wisconsin.

17. Rijnaarts, H.H.M., A. Bachmann, J.C. Jumelet, and A.J.B. Zehnder. 1990. Effect of desorption and intraparticle mass transfer on the aerobic biomineralization of ∝-hexachlorocyclohexane in a contaminated calcareous soil. Environ. Sci. Technol. 24:1349-1354.

18. Shimp, R. J. and R.L. Young. 1988. Availability of organic chemicals for biodegradation in settled bottom sediments. Ecotoxicol. Environ. Safety. 15:31-45.

19. Steen, W.C., D.F. Paris, and G.L. Baughman. 1980. Effects of sediment sorption on microbial degradation of toxic substances. *In* R.A. Baker (ed.), Contaminants and Sediments, Vol. 1, Ann Arbor Science, Ann Arbor, MI.

20. 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. 21:1201-1208.

21. van Ginkel, C.G., J.B. van Dijk, and A.G.M. Kroon. 1992. Metabolism of hexadecyltrimethylammonium chloride in *Pseudomonas* strain B1. Appl. Environ. Microbiol. 58:3083-3087.

22. Ventullo, R.M. and R.J. Larson. 1986. Adaptation of aquatic microbial communities to quaternary ammonium compounds. Appl. Environ. Microbiol. 51-356-361.

23. Weber, J.B. and H.D. Coble. 1968. Microbial decomposition of diquat adsorbed on montmorillonite and kaolinite clays. J. Agr. Food Chem. 16:475-478.

24. Weissenfels, W.D., H-J Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. Appl. Microbiol. Biotechnol. 36:689-696.

25. Xu, Shihe and S.A. Boyd. Unpublished data.

CHAPTER 3

ISOLATION AND PARTIAL CHARACTERIZATION OF TWO HDTMA-DEGRADING MICROORGANISMS

INTRODUCTION.

Quaternary ammonium compounds (QUATs) with alkyl chain lengths ranging from C_{12} to C_{18} are commercially valuable chemicals used in fabric softeners, disinfectants, hair care products, and detergents (22). These cationic surfactants are well known for their germicidal properties and exhibit toxicity at low (sub-mM) aqueous concentrations (2, 19) with alkyl chain length-dependent variations in toxicity. Despite their toxicity, the widespread use and discharge of QUATs into a variety of habitats have been met with the microbial adaptation to and mineralization of quaternary ammonium compounds by resistant, indigenous microbes. Time course biodegradability studies of dodecyltrimethyl-ammonium chloride (DTMAC) in lake water showed an initial lag of about 24 hours before surfactant mineralization, but subsequent additions of DTMAC were degraded immediately with no lag phase (19). A similar study using a model stream showed that adaptation plays a major role in surfactant degradation and successive exposure to QUATs results in increased degradative activity (16).

Several studies have focused on the biodegradation of specific quaternary ammonium compounds by pure and mixed microbial cultures. QUATs with alkyl chain lengths between C_9 and C_{18} appear to be susceptible to microbial attack. Ditallowdimethylammonium (DTDMA) disappearance in activated sludge reactors occurred by sorption

(precipitation) and biodegradation mechanisms (18). Hexadecyltrimethylammonium (HDTMA, C_{16}) was degraded by consortia of bacteria in sewage and soil (4) and in Ohio River water (16), and by a single bacterial strain isolated from sewage sludge (19). Additional studies have reported the degradation of decyltrimethylammonium, C_{10} (4), dodecyltrimethylammonium, C_{12} (17, 22), stearyl trimethylammonium, C_{18} and distearyldimethylammonium (6) among others (2).

Here, we report on the isolation and partial characterization of two bacterial species capable of using hexadecyltrimethylammonium as a sole carbon and energy source. We also provide information on the pathway of HDTMA degradation by these isolates.

MATERIALS AND METHODS.

Chemicals. n-Hexadecane and HDTMA-Br (99% purity) were obtained from Sigma Chemical, Co., St. Louis, MO. ^{14}C-HDTMA-Br (Moravek Biochemicals, Inc., Brea, CA, 55 mCi/mmol, 98% purity) was labelled in the C_{16} position of the hexadecyl chain, i.e. the carbon distal from the ammonium headgroup. ^{14}C-HDTMA-I (American Radiolabelled Chemicals, St. Louis, MO, 52 mCi/mmol) was methyl labelled and is referred to hereafter as [methyl-^{14}C]-HDTMA. All chemicals were used without further purification.

Isolation and maintenance of the microorganisms. A sample of activated sewage sludge was obtained from the Lansing, Michigan municipal wastewater treatment plant. Aliquots of the sludge were introduced into flasks containing a minimal medium [high-buffer broth (HBB) (8)] and n-hexadecane (433 uM) and HDTMA at varying concentrations of 10, 100, or 1000 uM. After an initial increase in turbidity, aliquots of the growing cultures were consecutively transferred (three times) to fresh HBB containing n-hexadecane and HDTMA. Following these transfers, an aliquot was inoculated into HBB containing HDTMA as the sole carbon and energy source. Upon an increase in turbidity, the culture was serially diluted and plated, and isolated colonies were streaked to purity. Solid isolation medium consisted of one-half strength nutrient broth (Difco) to which 1.5% agar was added. Cultures were maintained in HBB containing 500 uM HDTMA.

Assessment of ability to degrade HDTMA. Utilization of HDTMA as a sole carbon and energy source was determined in the following

mineralization assay. HDTMA solutions (in phosphate-buffered saline (PBS) containing 8.5 g NaCl, 0.3 g KH_2PO_4, and 0.6 g Na_2HPO_4 per liter of distilled water (Milli Q, Millipore Corp., Bedford, MA), pH 7.0) containing 1 ng mL^{-1} to 100 ug mL^{-1} HDTMA, including sufficient [chain-labelled-^{14}C]-HDTMA to attain initial activities of approximately 5,000 dpm mL^{-1}, were aseptically added to 150 mL glass serum bottles. Bottles were inoculated with washed, early stationary phase cultures of 101S or 102G at final densities of 10^8 cells mL^{-1} (A_{600}, Spectronic 88 spectrophotometer), crimp capped with Teflon-lined septa, and incubated at 25 °C on a rotary shaker (150 rpm). At predetermined intervals, 1 mL of the aqueous phase and 1 mL of the gaseous phase in the bottle were removed by syringe, transferred to a tube containing 1 mL 2 N HCl, and the evolved $^{14}CO_2$ was trapped on KOH-saturated filter paper suspended from the tube stopper in a plastic cup. Tubes sat overnight to allow quantitative recovery of $^{14}CO_2$. Filter papers were transferred to glass scintillation vials containing 7.5 mL Safety-Solve scintillation counting cocktail for aqueous solutions (Research Products International Corp., Mt. Prospect, IL). The vials were allowed to sit overnight in the dark to reduce chemiluminescence and total radioactivity was determined by liquid scintillation counting (LSC) on a Packard 1500 TriCarb liquid scintillation analyzer (Packard Instrument Co., Downer's Grove, IL) using external standard quench correction.

Additionally, a tandem experiment was conducted to assess the utilization of the terminal carbon atom on the hexadecyl chain and those in the three methyl groups adjacent to the ammonium headgroup. Glass serum bottles contained 75 mL PBS and HDTMA ranging from 100 ng mL^{-1} to

1 ug mL^{-1} with sufficient ^{14}C-HDTMA (methyl or chain-labelled) to attain initial activities of approximately 4,000 dpm mL^{-1}. Washed, early stationary phase HDTMA-induced cells (isolate 102G) were resuspended in PBS and inoculated into the bottles to a final density of 10^8 cells mL^{-1}. At precisely timed intervals, subsamples were withdrawn from the bottles as discussed above for determination of $^{14}CO_2$ produced from the two ^{14}C-labelled positions.

Toxicity assays. HDTMA-grown cells were exposed to various HDTMA concentrations in toxicity experiments designed to test the isolates' tolerance to HDTMA. Early stationary phase cells were collected by centrifugation and resuspended to a cell density of approximately 2 x 10^8 mL^{-1}. One mL of the cell stock was added to tubes containing 1 mL of HDTMA solutions of varying concentrations which, after dilution, resulted in HDTMA concentrations of 0.05, 0.1, 0.5, 0.75, 1, and 10 mM and a final cell density of 10^8 mL^{-1}. Suspensions were statically incubated for one hour before being serially diluted and plated onto one-half strength nutrient agar. Plate counts of the inoculum at the start of the experiment and of the incubated suspensions were used to determine the total percentage of viable cells after HDTMA exposure.

Negative staining/TEM analysis. A drop of a bacterial suspension of either 101S or 102G grown in 500 uM HDTMA was placed onto parolodion and carbon coated copper grids. After one minute, excess solution was blotted and the cells were stained for 30 seconds using a 1.5% aqueous ammonium molybdate solution after which the excess stain was removed. After air-drying for five minutes, the grids were observed and

photographed using a JEOL 100CX II Transmission Electron Microscope (Japan Electron Optics Laboratory) operated at 80 kV.

Biolog/FAME analysis. The substrate utilization patterns of both bacterial isolates were analyzed by the Biolog procedure (Biolog, Inc., Hayward, CA). This method tests the ability of microorganisms to utilize 95 different carbon sources and yields a "metabolic fingerprint" of the organism which is compared to microbes in Biolog's Microlog reference library. The lipid profiles of each organism were analyzed by gas chromatography after methyl esterification. The results of each analysis were cross-referenced in FAME's MIS standard library to provide a tentative identification of the organism. Similarity coefficients range from 0 to 1 and a coefficient greater than 0.6 is generally regarded as a good match to a reference microorganism.

Constitutive expression of responsible enzyme(s). To determine whether HDTMA degradation was a constitutive or inducible property of the isolates, cells were grown in non-selective media and assayed for HDTMA degradation. Isolates 101S and 102G were transferred three consecutive times over the course of one week in either a one-half strength nutrient broth solution or in HBB containing 1 g L^{-1} glucose as the sole carbon source. Early stationary phase cells were harvested by centrifugation (6500 x *g*, 10 minutes) and introduced (at a cell density of 10^8 mL^{-1}) into 75 mL PBS containing 120, 365, or 720 ng mL^{-1} HDTMA with [chain-labelled-^{14}C]-HDTMA to achieve an initial activity of approximately 4,000 dpm mL^{-1}. Subsamples were withdrawn from the bottles at precisely timed intervals and $^{14}CO_2$ production was monitored

as described above to determine if the enzymes responsible for HDTMA degradation were constitutively expressed.

Protein analysis. Both isolates were grown in one-half strength nutrient broth and in HBB/HDTMA (500 uM) and were harvested in early stationary phase (10^8 to 10^9 cells mL^{-1}) by centrifugation. Cell pellets were resuspended in a minimal amount of PBS and lysed by passage two times through a French Pressure cell press operated at 16,000 psi. Whole cells and cell fragments were separated from cell extracts by centrifugation (5,000 x *g*, 10 minutes). Proteins and molecular weight markers (Biorad) in the crude cell extracts were separated by SDS/polyacrylamide gel electrophoresis using the method of Laemmli (14), and were stained with Coomassie brilliant blue to allow visual comparisons.

Substrate utilization assays. Experiments were set up to determine the range of QUATs which could be utilized as sole carbon and energy substrates by the two isolates. 10, 100, and 500 uM solutions of the following were prepared in sterile HBB: methylamine, dimethylamine, trimethylamine, tetramethylammonium, nonyltrimethylammonium (NTMA, C_9), dodecyltrimethylammonium (DdTMA), dioctadecyldimethylammonium (DODMA), and N-(1-naphthyl)-ethylenediamine dihydrochloride. HDTMA solutions were also prepared to demonstrate metabolic activity and growth at the concentrations tested. Additionally, the cultures were inoculated into HBB containing 9.4 mg mL^{-1} hexadecanoic acid to aid in elucidation of the pathway of HDTMA degradation. Tubes were inoculated with washed, early stationary phase 101S or 102G cells at a density of 10^7 mL^{-1}, sealed with Parafilm, and incubated at 25 °C on a shaker (150 rpm).

Growth was measured by an increase in visible turbidity as well as an increase in optical density measurements (A_{600}) taken at 0, 24, and 48 hours.

Hydrophobicity assays. Assays were conducted to determine the cell hydrophobicity coefficients. A suspension of Octyl Sepharose CL-4B gel (Pharmacia Fine Chemicals, Uppsala, Sweden), diluted 1:2 in PBS, was pipetted to a length of approximately 3" into a 5 3/4" Pasteur pipet column with a glass wool plug. After settling, the column was rinsed three times with PBS. A ^{14}C-labelled cell suspension was obtained by harvesting [chain-labelled-^{14}C]-HDTMA grown cells of 101S or 102G, washing and resuspending to a density of 1 x 10^8 cells mL^{-1} in PBS. One mL of the cell suspension was added to a glass scintillation vial containing 7.5 mL scintillation cocktail to determine the total activity of the ^{14}C-labelled cells and another 1 mL aliquot of the cell stock was added to the column. Six eluent fractions of 1 mL PBS were collected in glass scintillation vials with counting cocktail and analyzed by LSC. The cumulative activity eluted was determined and the hydrophobicity coefficient (H.C.) was calculated as follows:

$$H.C. = \log \frac{\text{fraction of cells retained on gel}}{\text{fraction of cells eluted}} \qquad (1)$$

Calculated coefficients with negative values are indicative of cells with a hydrophilic surface; assays performed on hydrophobic cells result in hydrophobicity coefficients greater than zero.

RESULTS.

Two bacterial strains capable of using hexadecyltrimethylammonium bromide as a sole carbon and energy source were isolated from an activated sewage sludge sample using a batch enrichment culture technique. The two isolates, designated 101S and 102G, are Gram negative, oxidase positive, catalase positive, non-sporulating rods. When grown on one-half strength nutrient agar, colonies of 101S are tan with smooth edges; those of 102G are light tan with wavy edges and aging colonies have a slight granular appearance. Calculated hydrophobicity coefficients of -0.537 and -0.059 for 101S and 102G, respectively, indicate hydrophilic cell surfaces.

Transmission electron micrographs of negatively-stained cells are shown in Figures 3.1-3.5. Figures 3.1 and 3.2 depict a single cell of isolate 101S embedded in a crystalline structure of unknown origin and composition after growth in HBB with 500 uM HDTMA. Individual cells of 101S from a different culture are shown in Figure 3.3; a sex pilus is clearly visible between two cells. Figure 3.4 shows a cell of 101S with filamentous particles extending outward from the cell surface. Both isolates produce these chains which may be a result of HDTMA interaction with the cell surface, thereby causing sloughing of cellular material. A cluster of the larger 102G cells is seen in Figure 3.5. Unusual circular objects are seen clustered around these cells and their origin and nature are unknown.

Based on reference library searches following FAME and Biolog analyses, both isolates were tentatively identified as several

Figure 3.1. Transmission electron micrograph of HDTMA-degrading isolate 101S embedded in a crystalline matrix (10,000X magnification).

Figure 3.2. Transmission electron micrograph of isolate 101S surrounded by crystalline structure (10,000X magnification).

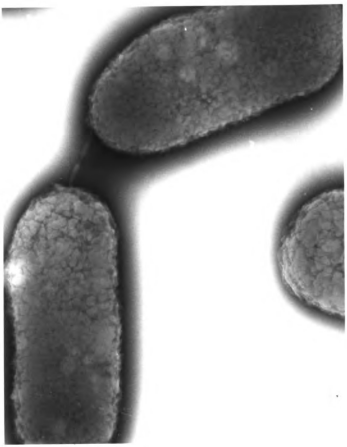

Figure 3.3. Transmission electron micrograph of HDTMA-degrading isolate 101S showing a sex pilus (36,000X magnification).

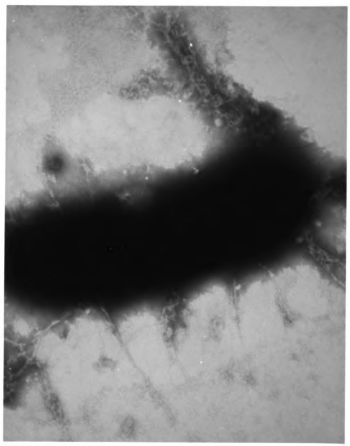

Figure 3.4. Transmission electron micrograph of isolate 101S with sloughed cellular material (29,000X magnification).

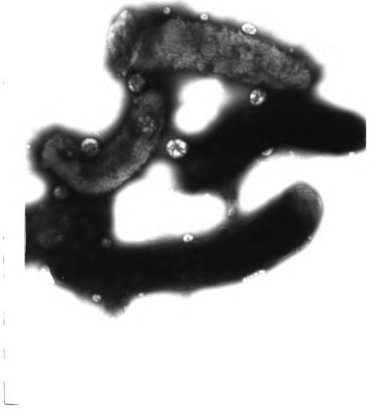

Figure 3.5. Transmission electron micrograph of isolate 102G with unusual circular objects adjacent to the cells (19,000X magnification).

microorganisms as shown in Table 3.1. The highest similarity coefficient obtained from several replicate analyses is listed for each tentative identification.

Table 3.1. Tentative identification of isolates 101S and 102G by FAME and Biolog analyses.

Isolate	Tentative Organism	Similarity Coefficients FAME	BIOLOG
101S	*Pseudomonas fluorescens* C		0.537
	P. aeruginosa	0.088	
	P. alcaligenes	0.077	
	P. pseudoalcaligenes	0.061	
	P. putida	0.033	
	Chryseomonas luteola	0.061	
102G	*Pseudomonas fluorescens* C		0.519
	P. putida biotype A	0.131	
	P. aeruginosa	0.086	
	P. alcaligenes	0.074	
	P. cepacia		0.051
	Enterobacter gergoviae		0.136

Based on the relatively high similarity coefficients obtained in the Biolog procedure, combined with the tentative identifications given by the FAME analyses, 101S and 102G are most likely *Pseudomonas* species.

HDTMA utilization by both isolates was evidenced by 1) an increase in turbidity upon inoculation into HBB media with HDTMA as the sole carbon and energy source, 2) the production of $^{14}CO_2$ from $^{14}C_{16}$-labelled compound, and 3) the disappearance of the initial foam in the HDTMA-containing media after culture growth. Figure 3.6 shows typical growth curves for both organisms in 500 uM HDTMA. At t_o, densities were 4.1 x 10^7 and 6.6 x 10^7 cells mL^{-1} for 101S and 102G, respectively. Plate

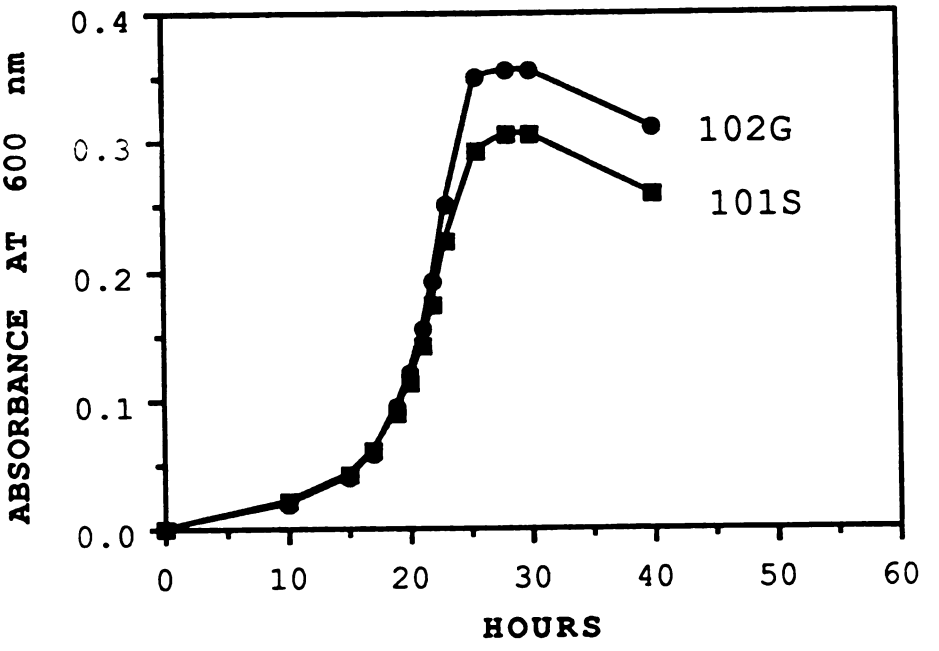

Figure 3.6. Growth curves for 101S and 102G in 500 uM HDTMA.

counts of both cultures during stationary phase showed that the densities had increased to 8.8 x 10^8 and 1.75 x 10^9 cells mL^{-1} for 101S and 102G, respectively, confirming growth on HDTMA. Abs_{600} readings of 0.065 and 0.119 correspond to approximately 10^8 cells mL^{-1} for 101S and 102G, respectively, indicating that 102G cells are larger than those of the other isolate (3 um x 0.6 um for 102G and 2 um x 0.8 um for 101S). At a cell density of 10^8 mL^{-1}, first-order mineralization kinetics were observed at aqueous HDTMA concentrations of 1 ug mL^{-1} or less (Figure 3.7). At concentrations greater than this, a lag period was observed prior to mineralization. With increasing initial HDTMA concentrations, the lag period also increased. Under these experimental conditions, typically 60 to 80% of the added HDTMA was recovered as CO_2 with the remainder presumably converted to biomass.

The isolates' ability to utilize amines and quaternary ammonium compounds with varying alkyl chain lengths was assessed. In addition to HDTMA, both 101S and 102G grow abundantly on hexadecanoic acid and dodecyltrimethylammonium (DdTMA), the 12-carbon QUAT similar in structure to HDTMA. Biolog assays revealed that both isolates also metabolize Tween 40 and Tween 80, two non-ionic surfactants. Methylamine, dimethylamine, trimethylamine, tetramethylammonium, nonyltrimethylammonium (NTMA), dioctadecyltrimethylammonium (DODMA), and N-(1-naphthyl) ethylenediamine dihydrochloride did not support growth at the concentrations tested.

To elucidate the possible sites of microbial attack during HDTMA degradation, mineralization time courses were completed for the differently ^{14}C-labelled HDTMA molecules at concentrations of 120 to 730

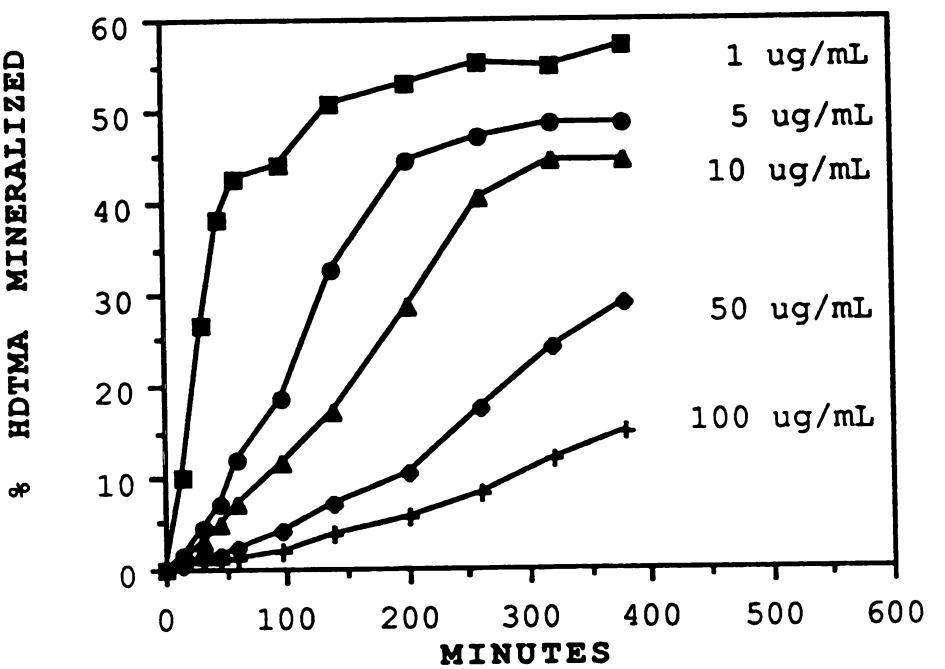

Figure 3.7. Mineralization time courses for varying HDTMA concentrations with 10^8 cells mL^{-1}.

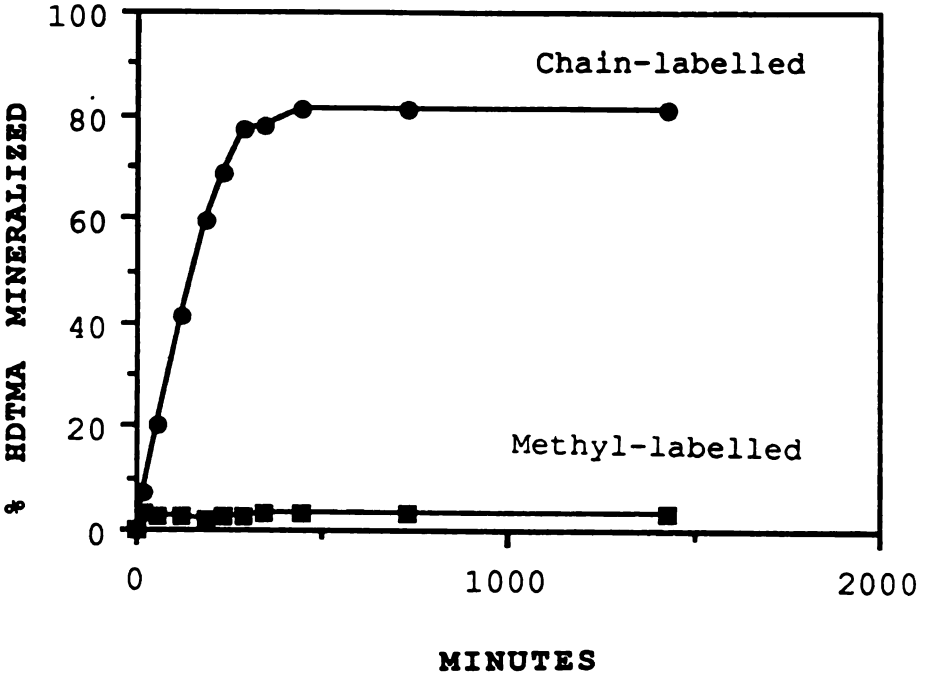

Figure 3.8. Mineralization curves showing release of $^{14}CO_2$ from methyl-labelled carbon and the terminal (C_{16}) position of the hexadecyl chain at initial HDTMA concentrations of 120 ng mL^{-1} inoculated with 10^8 cells mL^{-1} 102G.

ng mL^{-1}. For [methyl-^{14}C]-HDTMA, only approximately 1 to 3% of the labelled carbon was recovered as $^{14}CO_2$ after a 24-hour incubation. Conversely, approximately 80% of the ^{14}C-HDTMA labelled at C_{16} of the alkyl chain was converted to $^{14}CO_2$ in the same time period (Figure 3.8). Nitrogen free HBB was prepared to determine whether the isolates could utilize HDTMA as a sole nitrogen source. Both isolates grew to visible turbidity in this media for two transfers, but subsequent transfers of the cultures produced no significant growth. This implies that the nitrogen of the ammonium headgroup is not utilizable by the isolates and that nitrogen became a growth-limiting nutrient.

Quaternary ammonium compounds, such as HDTMA, are toxic to many microorganisms, yet 101S and 102G were capable of growth in 500 uM HDTMA. A survivability assay was conducted to study the acute toxic effects of HDTMA on the two isolates despite their apparent resistance to its effects. Figure 3.9 shows the percent survival of the organisms at HDTMA concentrations ranging from 0 to 10 mM. Generally, about 50% of the added cells were still viable after a one-hour incubation in 50 uM to 10 mM HDTMA with similar sensitivities exhibited by both 101S and 102G. Interestingly, while both organisms can mineralize HDTMA and grow to abundance in basal mineral solutions with initial HDTMA concentrations of up to 500 uM, HDTMA still exerts an initial, short-term toxicity from which the population must rebound prior to HDTMA mineralization.

Differing results were observed in induction studies of nutrient broth- versus glucose-grown cells of 101S and 102G. Upon inoculation into solutions of 120, 365, or 720 ng mL^{-1} HDTMA, HDTMA was mineralized

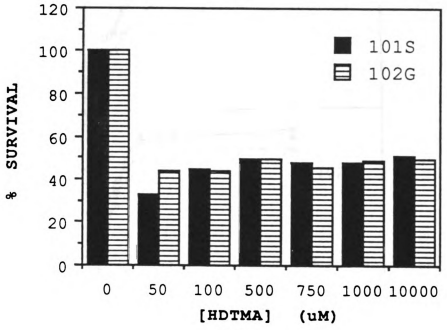

Figure 3.9. Survivability of isolates 101S and 102G incubated for one hour in HDTMA solutions of varying concentrations and a cell density of 10^8 cells mL^{-1}.

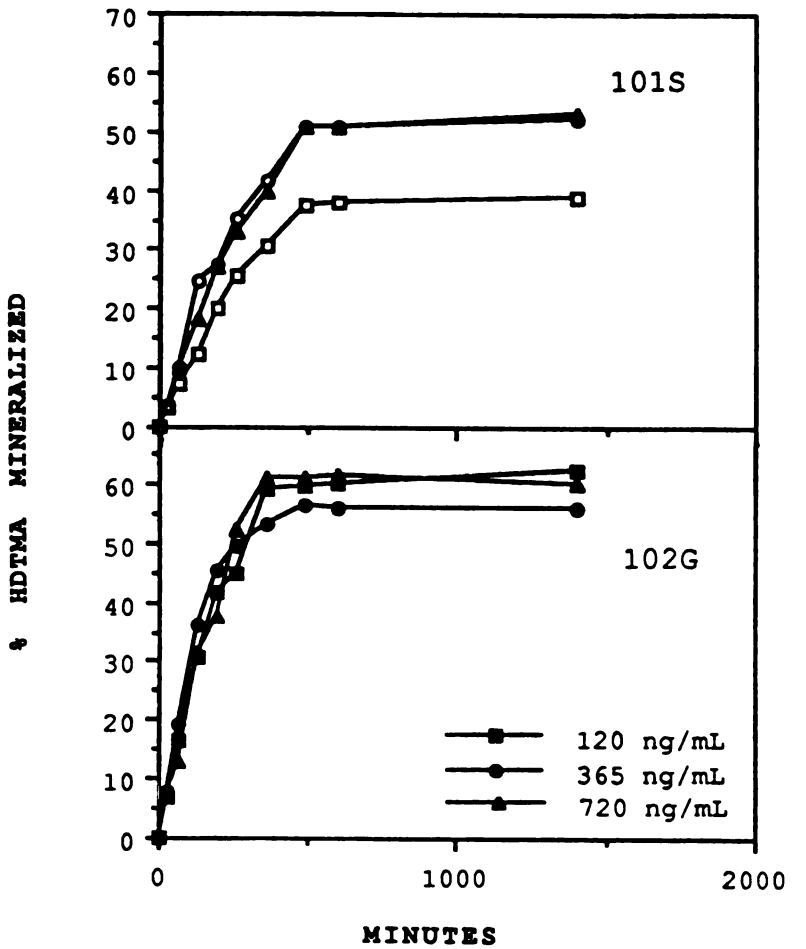

Figure 3.10. Mineralization curves for nutrient broth grown cells inoculated at a density of 10^8 mL^{-1} in various HDTMA solutions.

at first order rates by nutrient broth-grown cells at a cell density of 10^8 mL^{-1}, implying constitutive expression of the HDTMA-degradative enzymes (Figure 3.10). Kinetics analysis of the mineralization data revealed that rates of HDTMA mineralization by nutrient broth-grown cells approximated those observed for HDTMA-grown cells. For example, HDTMA mineralization rates for isolate 102G grown in HDTMA (nutrient broth) were: 8.36 (9.78), 3.08 (3.58), and 5.47 (8.31) (x 10^{-7} ng $cell^{-1}$ min^{-1}) for initial HDTMA concentrations of 120, 365, and 720 ng mL^{-1}, respectively. Conversion of HDTMA to CO_2 reached approximately 50 to 60%, slightly less than the mineralization observed for HDTMA-induced cultures. HDTMA mineralization by glucose-grown cells was initiated upon inoculation, but curves were sigmoidal in shape suggesting that cells were initially enzyme- or biomass-limited (Figure 3.11). This limitation was more obvious in isolate 102G as mineralization proceeded linearly for the first 500 minutes of incubation, followed by a period of rapid mineralization. Maximum percentage of the HDTMA mineralized by both cultures (40 to 60%) fell somewhat below the levels (60 to 80%) observed for HDTMA-induced cultures.

Electrophoresis of the cell extracts on SDS/polyacrylamide gels indicated that slightly different proteins are expressed by both isolates 101S and 102G (Figure 3.12). 101S had proteins of approximately 33,600 and 51,000 daltons which were absent in 102G. Also nutrient broth-grown cells had a protein band equivalent to approximately 89,000 daltons which was not visible in HDTMA-induced cells. In general, nearly identical proteins were observed for the two isolates

when induced for HDTMA degradation or when grown in the presence of a rich carbon supply (nutrient broth).

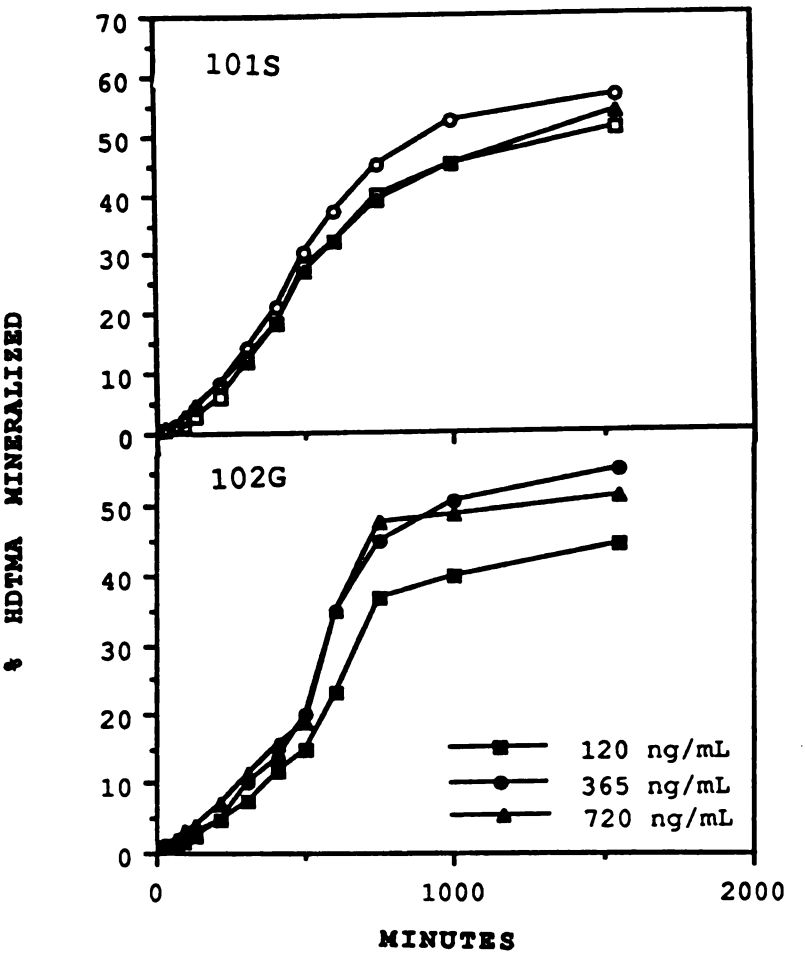

Figure 3.11. Mineralization curves for glucose grown cells inoculated at a density of 10^8 mL^{-1} in various HDTMA solutions.

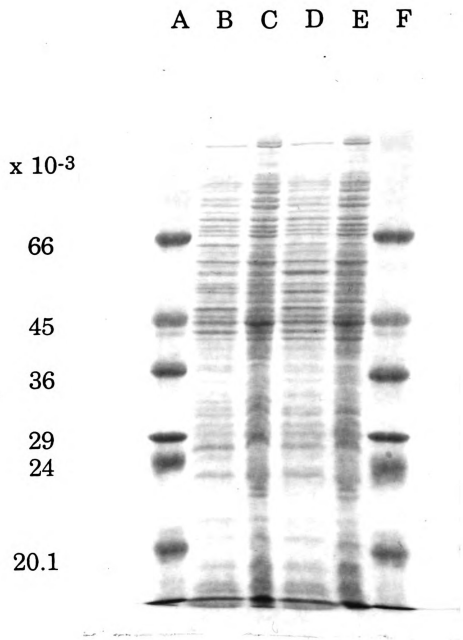

Figure 3.12. SDS/polyacrylamide gel produced from protein electrophoresis of cell extracts from 101S and 102G. Lanes A and F represent molecular weight markers (in daltons). Lanes B and D correspond to HDTMA-grown cells of 101S and 102G, respectively. Nutrient broth grown cells of 101S and 102G are presented in lanes C and E, respectively.

DISCUSSION.

The biochemical mechanisms by which surface active agents (like HDTMA) exert their toxicity on bacteria have been the focus of much research. There may be a variety of actual mechanisms by which this toxicity is manifested and the route may be dependent on both the organism and the surfactant concentration. Generally, the cause of the toxicity has been attributed to the physical properties of the cations and their binding interactions with the bacterial cell lipids (1, 9, 11) and membrane proteins (12). N-alkyl chain length determines the overall toxicity of these agents to bacteria (7) with anti-microbial activity maximized for substituent chain lengths of 14 to 16 carbon atoms (5). Gilbert et al. (7) proposed that this increased activity is a result of 1) dimerization of long-chain QUATs thereby enhancing cell membrane penetration or 2) dual binding sites on the cell surface for the larger moieties.

Until recently, isolation of HDTMA-degrading bacteria has been precluded by the QUAT's toxicity to many microorganisms. Previous reports (21) have described HDTMA degradation in chemostats, but not in batch cultures because of the high initial substrate concentrations and resultant toxicity. van Ginkel et al. (20) have reported the isolation of *Pseudomonas* strain B1 capable of using HDTMA as a sole growth substrate in batch cultures containing silica gel. Silica gel acted as an adsorbent to reduce the free HDTMA concentration and alleviate its toxicity. The two isolates described here, on the other hand, are capable of growth on HDTMA at high aqueous concentrations (500 uM) in

the absence of sorbent. Both strains are also capable of utilizing HDTMA sorbed to clay minerals (by cation exchange) or soils (15).

101S and 102G have been tentatively identified as *Pseudomonas*, although the exact species are unknown due to differing results obtained from Biolog and FAME identification procedures for each organism. Similarity coefficients of > 0.5 for *P. fluorescens* were observed for the Biolog procedure, but FAME analyses did not show any similarity between 101S or 102G and *P. fluorescens*. FAME similarity coefficients were all low (< 0.14) indicating low correlations between the isolates and reference microorganisms. It was not surprising that 101S and 102G were not better matched to reference organisms as both procedures were originally instituted to identify medically-important microbes and their applicability to environmental species is limited.

These two isolates are able to grow abundantly in concentrated HDTMA solutions (500uM), however, they are not totally immune to its toxic effects. Inoculation of HDTMA-induced cells of 101S or 102G into a 50 uM HDTMA solution will render up to 50% of the cells non-viable after a one-hour incubation. Interestingly, a 10 mM solution (with an HDTMA concentration 200 times greater) still results in about 50% survival of the cultures. The protective mechanisms allowing survival in and utilization of such high concentrations of HDTMA (500uM) are only speculative. The hydrophilic cell surfaces of the two isolates may play a role in offering some resistance to the mechanisms by which HDTMA exerts its toxicity. Membrane hydrophilicity may attract solution phase HDTMA to the cell surface where it can exchange with membrane-associated cations. In this manner, the HDTMA may be less toxic and more readily

available for uptake and utilization by the cells. In an electron micrograph of 101S (Figure 3.4) (and also seen in 102G cultures), it appears that cell material is being sloughed off of the cell membrane. In other micrographs of 101S (Figures 3.1 and 3.2) unique crystalline structures are formed around the cells, possibly from a metabolic by-product or in response to the toxicity of the HDTMA. The crystals may also be formed from solution phase HDTMA in the growth media as the cell serves as a nucleus for crystallization. The described crystals were only observed in one culture of organism 101S and subsequent attempts to observe the phenomenon were unsuccessful.

The pathways of degradation for HDTMA are speculative and differing theories have been proposed by several researchers. van Ginkel et al. (20) studied the metabolism of HDTMA by *Pseudomonas* strain B1 and suggested that the molecule was first attacked at the C-N bond, thereby splitting it into trimethylamine and hexadecanal which was oxidized to hexadecanoic acid and subsequently to CO_2, H_2O, and biomass by β-oxidation. The trimethylamine produced by the cleavage was not further oxidized by the isolated strain and accumulated stoichiometrically in the system. Dean-Raymond and Alexander (4) studied the pathway of mineralization for decyltrimethylammonium (DTMA) and differ from van Ginkel in their theory of QUAT degradation. Analysis of metabolic by-products led these authors to propose a biodegradation pathway involving oxidation of the terminal carbon of the long alkyl chain of the QUAT followed by β-oxidation of the chain, ultimately to carboxymethyltrimethylammonium. While our study did not comprehensively address the metabolic pathway of HDTMA degradation, we

found that 101S and 102G are 1) unable to utilize HDTMA as a sole nitrogen source, 2) unable to significantly utilize the methyl carbons of the ammonium headgroup, 3) able to use hexadecanoic acid as a growth substrate, and 4) unable to use trimethylamine as a growth substrate. Tandem mineralization studies revealed that approximately 60 to 80% of the terminal carbon on the hexadecyl chain is mineralized in a few hours, while only about 1 to 3% of the methyl carbons are converted to CO_2 over the course of 24 hours. Combined, these results may suggest that the two described isolates metabolize HDTMA according to the pathway proposed by van Ginkel et al. (20).

Attempts to isolate plasmids from the isolates by the method of Hirsch et al. (10) were unsuccessful, suggesting that the genes coding for HDTMA degradation were located on chromosomes rather than on a transferable plasmid. Induction studies of the isolates after three consecutive transfers in nutrient broth over the course of a week showed that cells retained their ability to degrade HDTMA at first-order rates, implying that the enzymes responsible for HDTMA degradation were either constitutively expressed or had long half-lives (Figure 3.10). However, additional observations suggested that these enzymes were inducible rather than constitutive and may be plasmid-borne. Sigmoidal HDTMA mineralization curves were observed for cells grown in glucose for three consecutive transfers in one week when inoculated into HDTMA solutions (Figure 3.11). Glucose-grown cells exhibited an initially linear phase of HDTMA mineralization, followed by a period of rapid mineralization. These results suggested that HDTMA-degradative enzymes may be inducible rather than constitutive and may be plasmid-borne. Possibly, only a

small percentage of the microbial population retained the plasmids coding for the enzymes when grown in glucose. Consequently, upon inoculation into the HDTMA-containing media, some HDTMA mineralization occurred while plasmid transfer to all cells continued, eventually initiating rapid HDTMA mineralization, and hence, sigmoidal mineralization curves (Figure 3.11). Conjugation between two bacterial cells (as shown in Figure 3.3) involves transfer of genetic material and implies the presence of large plasmids (3). The plasmid encoding for HDTMA degradation is likely to be large due to 1) the complexity of the HDTMA molecule, 2) the additional need to code for resistance to its toxic effects, and 3) the evidence of conjugation in isolate 101S. Often, these larger plasmids are more difficult to isolate and our unsuccessful attempts do not preclude the presence of plasmids in the isolates. Possibly, some constitutent in the complex nutrient broth resembles HDTMA, thereby causing 101S and 102G to retain their HDTMA-degrading enzymes even after repeated transfers in the absence of HDTMA. However, after growth on a simple substrate (glucose), a portion of the bacterial population lost the genetic capability to degrade HDTMA which required a period of induction before rapid HDTMA mineralization was observed.

REFERENCES

1. Blois, D.W. and J. Swarbrick. 1972. Interaction of quaternary ammonium bactericides with biological materials I: Conductometric studies with cephalin. J. Pharmaceut. Sci. 61:390-399.

2. Boethling, R.S. 1984. Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants. Water Res. 18:1061-1076.

3. Brock, T.D. and M.T. Madigan. 1988. Biology of Microorganisms. 5th ed., Prentice-Hall, Englewood Cliffs, N.J.

4. Dean-Raymond, D. and M. Alexander. 1977. Bacterial metabolism of quaternary ammonium compounds. Appl. Environ. Microbiol. 33:1037-1041.

5. Dubois, A.S. and E. Valko. 1945. Correlation between anti-bacterial power and chemical structures of higher alkylammonium ions. J. Bacteriol. 50:481-489.

6. Federle, T.W. and R.M. Ventullo. 1990. Mineralization of surfactants by the microbiota of submerged plant detritus. Appl. Environ. Microbiol. 56:333-339.

7. Gilbert, P. and A. Altaae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Letts. Appl. Microbiol. 1:101-104.

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

9. Hansch, C. and J. Clayton. 1973. Lipophilic character and biological activity of drugs II: the parabolic case. J. Pharmaceut. Sci. 62:1-43.

10. Hirsch, P.R., M. Van Montagu, A.W.B. Johnston, N.J. Brewin, and J. Schell. 1980. Physical identification of bacteriogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120:403-412.

11. Isomaa, H., H. Bergman, and P. Sandberg. 1979. The binding of CTAB, a cationic surfactant to the rat erythrocyte membrane. Acta Pharmacol. et Toxicol. 44:36-42.

12. Kondo, T. and M. Tomizawa. 1969. Mechanism of hemolysis by cationic surface active agents. J. Pharamaceut. Sci. 58:1378-1381.

13. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cationic surfactants in laboratory and environmental systems. Dev. Ind. Microbiol. 24:425-434.

14. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 22:680-685.

15. Mueller, S.A., W.F. Guerin, and S.A. Boyd, Chapter 1, unpublished data.

16. Shimp, R.J., B.S. Schwab, and R.J. Larson. 1989. Adaptation to a quaternary ammonium surfactant by suspended microbial communities in a model stream. Environ. Toxicol. Chem. 8:723-730.

17. Shimp, R.J. and R.L. Young. 1988. Availability of organic chemicals for biodegradation in settled bottom sediments. Ecotoxicol. Environ. Safety. 15:31-45.

18. Sullivan, D.E. 1983. Biodegradation of a cationic surfactant in activated sludge. Water Res. 17:1145-1151.

19. Tubbing, D.M.J. and W. Admiraal. 1991. Inhibition of bacterial and phytoplanktonic metabolic activity in the lower Rhine River by ditallowdimethylammonium chloride. Appl. Environ. Microbiol. 57:3616-3622.

20. van Ginkel, C.G., J.B. van Kijk, and A.G.M. Kroon. 1992. Metabolism of hexadecyltrimethylammonium chloride in *Pseudomonas* strain B1. Appl. Environ. Microbiol. 58:3083-3087.

21. van Ginkel, C.G. and M. Kolvenbach. 1991. Relations between the structure of quaternary alkyl ammonium salts and their biodegradability. Chemosphere. 23:281-289.

22. Ventullo, R.M. and R.J. Larson. 1986. Adaptation of aquatic microbial communities to quaternary ammonium compounds. Appl. Environ. Microbiol. 51-356-361.

APPENDIX

APPENDIX A

HEXADECYLTRIMETHYLAMMONIUM: