This is to certify that the

thesis entitled
BIOCOMPATIBILITY OF QUATERNARY AMMONIUM CATIONS USED TO ENHANCE THE IMMOBILIZATION OF NONIONIC ORGANIC CONTAMINANTS IN SOILS

presented by
JEFFREY VERNE NYE

has been accepted towards fulfillment
of the requirements for

M.S. degree in CROP AND SOIL SCIENCES

Major professor

Date 12/17/92

BIOCOMPATIBILITY OF QUATERNARY AMMONIUM CATIONS USED TO ENHANCE THE IMMOBILIZATION OF NONIONIC ORGANIC CONTAMINANTS IN SOILS

By

Jeffrey Verne Nye

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Crop and Soil Sciences

1993

ABSTRACT

BIOCOMPATIBILITY OF QUATERNARY AMMONIUM CATIONS USED TO ENHANCE THE IMMOBILIZATION OF NONIONIC ORGANIC CONTAMINANTS IN SOILS

By

Jeffrey Verne Nye

An *in situ* soil modification technology utilizing quaternary ammonium cations (QACs) to increase sorptive capacity toward nonionic organic contaminants coupled with biodegradation of sorbed pollutants has been investigated. We evaluated the biocompatibility of QACs, primarily hexadecyltrimethylammonium (HDTMA), to contaminant-degrading microorganisms. Biodegradation of added ^{14}C-substrates in HDTMA-treated soils indicated an increased lag in response to mineralization with increased levels of HDTMA treatment. Equivalent amounts of HDTMA prebound to soil or clay had little adverse effect on mineralization. Dioctadecyldimethylammonium (DODMA) added to soil at 50% of the CEC had no significant effect on mineralization of salicylate and naphthalene. Characterization of eleven bacterial isolates from an HDTMA-treated soil indicated *Bacillus* to be the predominant bacterial type. HDTMA was found to be highly toxic to *Pseudomonas putida* (ATCC 17484), but additions of small amounts of clay alleviated this toxicity. DODMA was observed to be less toxic to *Pseudomonas putida* (ATCC 17484).

To my Fiance', Renee' Ann Richmond, for her love and support throughout my graduate studies, and to my parents for instilling in me the importance of an education.

ACKNOWLEDGEMENTS

The author would like to thank his major advisor, Dr. Stephen Boyd, for giving him the opportunity to continue his education, and for the continual assistance he has provided. The author also wishes to extend his vast appreciation to Dr. William Guerin for contributing a large amount of time and effort to his studies. He also would like to acknowledge Dr. Craig Criddle and Dr. James Tiedje for their participation on his committee, Dr. James Crum for assistance in collection and analysis of soil samples, Dr. Fiona Crocker for aid in experimental design and interpretation of results, and Sherry Mueller for challenging him intellectually in classes and in the laboratory. Finally, the author would like to thank Renee' Ann Richmond, Michael Bennett II, his parents, and sisters for everything they have done for him.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Contamination of soils, subsoils and aquifer materials with Nonionic Organic Contaminants (NOCs) has become an everincreasing problem due to the widespread use of refined petroleum products and coal as a source of energy and power. Soils may become contaminated with NOCs through many processes, such as leaking storage tanks. Once present in soil, the movement and interactions of NOCs throughout the soil profile and biodegradation are two important factors that affect the ultimate fate of these types of contaminants. The fate of NOCs are important due to their inherent carcinogenic properties and the possibility for human exposure once present in groundwater, a large source of drinking water.

Movement of NOCs in soils and aquifer materials is dependant upon interactions with components of these materials. These interactions are widely variable, dependant upon the characteristics of the contaminants and soils. Water solubility, charge, and polarity are important characteristics of NOCs; clay mineralogy and content, percent organic matter, temperature and water content for soils (18, 21, 46, 11, 47, 48, 30, 31).

The interactions of organic compounds containing permanent positive charges such as diquat [6,7-dihydrodipyrido (1,2-a:2'1'-c)-pyrazidiinium dibromide] (40) and benzylamine, with a pKa of 9.33, (29)

22is dominated by ion exchange reactions onto chiefly clay mineral surfaces and inner layers, and to some extent organic matter, that possess negatively charged regions. This reaction removes the positively charged contaminants from the soil-water solution rendering them unavailable for plant uptake (14) , microbial degradation (40), toxicity (43), or to perform its meant task such as insecticidal properties (41). Organic, cationic compounds such as diquat and benzylamine produce highly nonlinear isotherms with a characterstic plateau at or near the cation exchange capacity of the soil, indicating saturation of negatively charged sites and have solute competition for these adsorption sites (42). These types of contaminants are characterized as having a very low potential for leaching.

Similarly, in soil systems containing a low moisture content, benzene and chlorobenzenes were shown to interact with clay minerals and soils through adsorption processes (10). These adsorptive interactions are prevalent in very dry soils, in soils suspended in relatively nonpolar organic solvents such as hexane or octanol (47, 10), or onto soils from the vapor phase with suppression by water as was shown to be the case for methylbromide (13).

In contrast, NOC interactions in soils and aquifer materials under water-saturated conditions is dominated by partitioning into the soil organic matter fraction (11, 12, 19, 20). It is thought that soil organic matter acts as a bulk organic phase, solubilizing NOCs and removing them from the soil-water solution (8, 9). The highly hydrophobic NOCs are able to become solubilized into the hydrophobic regions of the soil organic matter much like the partitioning of an

organic compound from water into octanol (8, 10, 11, 12, 22, 23, 24). This partitioning mechanism is characterized by low equilibrium heats of sorption, linear sorption isotherms over a broad solute concentration range and lack of solute competition in multisolute systems (8, 10, 11, 12).

The partitioning of a nonionic organic solute into an immiscible organic solvent such as octanol can be measured in terms of the solute's K_{ow}, or octanol-water partitioning coefficient. The partition coefficient for a given solute in soil can be normalized to the soil's organic matter content to give the K_{om}, which equals the measured partitioning coefficient divided by the fractional organic matter content [$K_{om}=K/f_{om}$] (9, 11). The relationship between the water solubilility of the solute and the K_{om} is identical to the K_{ow}, such that solutes with inherently low water solubilities will partition strongly with soil organic matter (9, 11).

Clay minerals are ineffective as sorbents for NOCs due to their highly hydrated state in soil water systems. Naturally occurring metal cations (Na^{+} and Ca^{2+}) are exchanged onto the surfaces and inner layers of clay minerals satisfying the cation exchange capacity. These inorganic cations have strong affinities for water and large spheres of hydration, inactivating the clay minerals as sorbents for the highly hydrophobic NOCs (17, 10).

For the reasons stated above, soils containing high amounts of organic matter are capable of immobilizing NOCs through partitioning. The problem arises in soils and aquifer materials that are inherently low in organic matter and unable to interact with and immobilize NOCs.

In these circumstances, NOCs are allowed to migrate through the soil profile to groundwater creating a substantial risk to the health of humans that use this as a clean water source.

A soil modification technology utilizing quaternary ammonium cations to enhance immobilization of NOCs has been suggested (4, 5). The soil modification process entails addition of a cationic surfactant of the form $[R\text{-}N^{+}(CH_3)_3]$ and $.[RR'\text{-}N^{+}(CH_3)_2]$, such as hexadecyltrimethylammonium bromide (HDTMA) where R is a C-16 alkyl chain, in an aqueous solution to soil downgradient of a mobile plume of NOCs. HDTMA interacts strongly with soil displacing naturally occurring inorganic cations from clay minerals satisfying the soil's cation exchange capacity and creating a stable organo-clay complex with increased sorption capacity for NOCs (5). Quaternary ammonium cations such as HDTMA adsorb to soils and clays stoichiometrically with the cation exchange capacity resulting in a nonlinear sorption isotherm containing a plateau at or near the soil cation exchange capacity (5). This modification technology transforms clay minerals from a hydrophilic entity to a very hydrophobic, organophilic environment for larger organic cations (5).

Boyd *et.al.* have shown that modified clays and soils have increased sorptive capacity for nonionic organic contaminants as indicated in sorption isotherms by the batch method. Partition constants increased by about 12 to 16 times for benzene, perchloroethene and dichlorobenzene in an HDTMA-modified A horizon soil as compared to the unmodified soil. Partition constants 200 times greater were observed for the same compounds on the HDTMA-modified B horizon soil as

compared to the unmodified B horizon soil (5).

Small organic cations such as tetramethylammonium, when used to modify clay minerals, do not increase the sorptive capacity to the extent that larger organic cations such as HDTMA can towards NOCs. Tetramethylammonium-smectite showed great uptake for benzene from water, however had little effect for substituted benzenes such as ethylbenzene indicating a surface adsorbent system (26, 27). It is thought that the organo-clays modified with relatively small organic cations such as tretramethylammonium bromide displace the highly hydrated inorganic cations creating adsorptive clays, binding NOCs through adsorptive interactions (17). Conversely, the larger organic cations such as HDTMA create an organic phase within the clay minerals into which organic pollutants may become solubilized, marking a partitioning mechanism (4, 5, 17).

Coupling the enhanced immobilization of NOCs through soil modification utilizing quaternary ammonium cations with biodegradation of sorbed pollutants is currently being studied. The question arises of the biocompatibility of the cationic surfactants. n-Alkyltrimethyl-ammonium bromides have been shown to have toxic effects on bacterial species at low uM concentrations (16). These types of cationic surfactants have bactericidal activity towards a wide array of bacteria, both gram positive and negative, as well as fungi and yeasts (6, 16, 33).

Gilbert and Al-Taae (16) observed a parabolic relationship between the alkyl chain length and toxicity, with n=14 to 16 resulting in the greatest toxicity. Chain lengths of less than 14 and greater than 16

were less toxic to the bacterial types tested. They attributed this to different binding sites and relative affinites for the various cations tested and also hypothesized that the n=14 and 16 cations were more likely to form dimers, producing a molecular species that is more suitable to cross the cellular membranes and interact with target molecules.

Beaubien and Jolicoeur (2) further added that not only is the hydrophobic nature of the alkyltrimethylammonium bromide cations important in relative toxicities, but the nature of the headgroup functions in the toxicity as well. Comparisons of analogous surfactants differing only in the headgroup, n-alkylpyridinium bromides and n-alkylcarboxylate bromides, showed the cationic surfactant to be 10-fold more toxic to bacteria. They noted a simple inhibition pattern in the heat flux constant at low concentrations, decreasing rapidly with increased surfactant concentration.

The effects of organic cations on aquatic microbial communities has been studied due to the large amounts of surfactants being released to streams and rivers. Approximately 3 x 10^6 to 4 x 10^6 metric tons of synthetic surfactants are produced yearly with a large percentage being released to aquatic environments (31, 44). Concentration values for surfactants in aquatic environments have been measured, ranging from 6 ug liter^{-1} for ditallowdimethylammonium chloride (DTDMAC) in the River Rhine at Lobith to 25 ug liter^{-1} in the River Meuse (39).

Federle and Ventullo (15) compared the microbial population and activity in a control pond and one in which large amounts of laundromat wastewater containing surfactants had been introduced over a long period

of time. An approximate two-fold increase in bacterial numbers and ATP content, whereas a two-fold reduction in FDA hydrolysis, was found to occur in the pond receiving the surfactant-containing wastewater. A reduction in the number of fungi and an increase in the number of bacteria accounted for the overall increase of activity. Tubbing and Admiraal (38) measured the effect of ditallowdimethylammonium chloride (DTDMAC) on bacterial and phytoplanktonic metabolic activity. Concentrations of 0.03 to 0.1 mg of DTDMAC liter^{-1} were found to significantly decrease the growth rate of bacterioplankton and photosynthetic rate of phytoplankton. They also recorded a dependance of DTDMAC toxicity on the concentration of suspended matter in the system by the differences in thymidine incorporation over several days of sampling where the amount of suspended material in the river changed. The adsorption of DTDMAC to suspended material was identified as the most likely reason for the reduced affect on heterotrophic bacteria.

Similarly, the effect of quaternary ammonium surfactants on a suspended microbial community in a model stream were assessed (35). Dodecyltrimethyl-ammonium chloride introduced into the model stream was found to have no significant impact on the total size of the microbial community. The ability of the microbial community to degrade the surfactant increased by 10- to 1,000-fold and after prolonged exposure to it they were able to develop resistance to "shock loads" of the surfactant. The adaptation to the surfactant was lost when introduction was inconsistent.

The production of a novel protein from a plasmid encoded gene responsible for quaternary ammonium cation resistance has been studied

by Tennent *et.al.* (36). They have isolated a *Staphylococcus aureus* strain which contains the plasmid pSK1 carrying the *qacA* determinant responsible for linked resistance to quaternary ammonium compounds. A 50kD protein, resulting from the plasmid, has been implicated as the factor affecting the resistance to quaternary ammonium cations. It is thought that this protein is located within the cellular membrane controlling the flux of organic cations into the cells.

The ability of microorganisms to degrade cationic surfactants has been shown to be widespread in nature. Natural environments such as streams receiving wastewater and manmade systems like sludges of wastewater treatment plants maintain active populations of cationic surfactant degrading microorganisms. The rates of biodegradation of soil- and sediment-bound cationic surfactants were found to be lower than soil free controls further indicating the unavailability of bound cations (36), whereas some researchers have found biodegradative rates in sediment-bound cationic surfactant systems to be rapid and complete (25).

Several hypotheses indicating the mode of toxicity of cationic surfactants have arisen. Cationic surfactants are thought to be surface-active agents able to disrupt bacterial cell membranes, causing internal macromolecules such as enzymes to be leaked into the exterior (7). Alternatively, HDTMA has been shown to bind to proteins embedded in the cell membrane as well as DNA and polysaccharides found within the cell rendering them nonfunctional (34). HDTMA also inhibits the catalytic activity of soluble and membrane-bound F_1 ATPases in a noncompetitive fashion (1).

REFERENCES

1. Barzu, O., F. Guerrieri, R. Scarfo, G. Capossa, and S. Papa. 1989. Effect of cetyltrimethylammonium on ATP hydrolysis and proton translocation in the F_0-F_1 H^+ ATP synthase of mitochondria. J. of Bioenergetics and Biomembranes 21:403-414.

2. Beaubien, A., L. Keita, and C. Jolicoeur 1987. Flow microcalorimetry investigations of the influence of surfactants on a hetergeneous aerobic culture. App. Environ. Micro. 53:2567-2573.

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

4. Boyd, S.A., M.M. Mortland, and C.T. Chiou. 1988. Sorption characterstics of organic compounds on hexadecyltrimethylammonium-smectite. Soil Sci. Soc. Am. J. 52:652.

5. Boyd, S.A., J.F. Lee, and M.M. Mortland. 1988. Attenuating organic contaminant mobility by soil modification. Nature. 333:3451.

6. Bull, H.B. 1947. Advan. Protein Chem. 3:95.

7. Cabral, J.P.S. 1991. Mode of antibacterial action of dodine (dodecylguanidine monoacetate) in *Pseudomonas syringae*. Can. J. Microbiol. 38:115-123.

8. Chiou, C.T., L.J. Peters, and V.H. Freed. 1981. Soil-water equilibria for nonionic organic compounds. Science. 213:684.

9. Chiou, C.T., D.W. Schmedding, and M. Manes. 1982. Partitioning of organic compounds in octanol-water systems. Environ. Sci. Technol. 16:4-10.

10. Chiou, C.T. and T.D. Schoup. 1985. Environ. Sci. Technol. 19:1196-1200.

11. 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.

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

13. Chisholm, R.C. and L. Koblitsky. 1943. Sorption of methylbromide by soil in a fumigation chamber. J. Econ. Entomol. 36:549-551.

14. Coats, G.E., H.H. Funderburk Jr., J.M. Lawrence, D.E. Davis. 1966. Factors affecting persistence and inactivation of diquat and paraquat. Weed Res. 6:58-66.

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

16. Gilbert, P. and A. Al-Taae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Letters in App. Micribiol. 1:101-104.

17. Jaynes, W.F. and S.A. Boyd. 1990. Trimethylphenylammonium-smectite as an effective adsorbent of water soluble aromatic hydrocarbons. J. Air Waste Manage. Assoc. 40:1649-1653.

18. Jaynes, W.F. and S.A. Boyd. 1991. Clay mineral type and organic compound sorption by hexadecyltrimethylammonium-exchanged clays. Soil Sci. Soc. Am. J.

19. Karickhoff, S.W. 1981. Semi-empirical estimation of sorption of hydrophobic pollutants on natural sediments and soils. Chemosphere. 10:833-846.

20. Karickhoff, S.W., P.S. Brown, and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13:241-248.

21. Karickhoff, S.W. 1984. Organic pollutant sorption on aquatic systems. J.Hydraul.Eng. 110:707-735.

22. Lambert, S.M., P.E. Porter, and H. Schieferstein. 1965. Movement and sorption of chemicals applied to soil. Weeds. 13:185-190.

23. Lambert, S.M. 1967. Functional relationship between sorption in soil and chemical structure. J. Agric. Food Chem. 15:572-576.

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

25. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cation surfactants in laboratory and environmental systems. Devl. Indus. Micro. 24:425-434.

26. 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. 34:1365.

27. Lee, J.F., M.M. Mortland, C.T. Chiou, D.e. Kile, and S.A. Boyd. 1990. Adsorption of benzene, toluene and xylene by two tetramethylammonium smectites having different charge densities. Clays and Clay Minerals. 38:113.

28. Marchesi, J.R., N.J. Russell, G.F. White, and W.A. House. 1991. Effects of surfactant adsorption and biodegradability on the distribution of bacteria between sediments and water in a freshwater microcosm. App. Environ. Micro. 57:2507-2513.

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

30. Pinck L.A., W.F. Holton, and F.E. Allison. 1961. Antibiotics in soils: I. physico-chemical studies of antibiotic-clay complexes. Soil Sci. 91:22-28.

31. Pinck L.A., D.A. Soulides, and F.E. Allison. 1961. Antibiotics in soils: II. extent and mechanism of release. Soil Sci. 91:94-99.

32. Saltzman, S., L. Kliger, and B. Yaron. 1972. Adsorption-desorption of parathion as affected by soil organic matter. J. Agric. Food Chem. 20:1224-1226.

33. Schulman, J.H. and E.K. Rideal. 1937. Proc. Roy. Soc., Ser. B. 122:29.

34. Scott, J.E. Methods in carbohydrate chemistry, Vol V, pp. 38-44. 1965. Ed. L.P.

35. 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. Tox. Chem. 8:723-730.

36. Shimp, R.J. and R.L. Young. 1987. Availability of organic chemicals for biodegradation in settled bottom sediments. Ecotox. and Environ. Safety. 15:31-45.

37. Tennant, J.M., B.R. Lyon, M. Midgley, G. Jones, A.S. Purewal, and R.A. Skurray. 1989. Physical and Biochemical Characterization of the qacA gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. J. Gen. Micro. 135:1-10.

38. Theng, B.K.G. The chemistry of clay-organic reactions, pp. 221-238 (Wiley, New York, 1974).

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

40. Van Leeuwen, K., C. Roghair, J. de Greef, and T. de Nijs. 1990. Wasverzachters. II. Resultaten van aanvullend onderzoek. Water 11:295-299.

41. 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.

42. Weber, J.B. and D.C. Scott. 1966. Science 152:1400.

43. Weber, J.B., P.W. Perry, and R.P. Upchurch. 1965. The influence of temperature and time on the adsorption of paraquat, diquat, 2,4-d and prometone by clays, charcoal and an anion-exchange resin. Soil Sci. Soc. Am. Proc. 29:678-688.

44. 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.

45. Werdelmann, B.W. 1984. Tenside in unserer Welte-heute und morgen. p. 3-21. *In* Procedding of the Second World Surfactants Congress. vol. 1. Syndicat National des Fabricants d'Agents de Surface et de Produits Auxiliaires Industriels. Paris.

46. Wszolek, P.C. and M. Alexander. 1979. Effect of desorption rate on the biodegradation of n-alkylamines bound to clay. J. Agric. Food Chem. 27:410-414.

47. Wu, S. and P.M. Gschwend. 1986. Sorption kinetics of hydrophobic organic compounds to natural sediments and soils. Environ.Sci.Technol. 20:717-725.

48. Yoron, B. and S. Saltzman. 1972. Influence of water and temperature on adsorption of parathion by soils. Soil Sci. Soc. Amer. Proc. 36:583-587.

Biodegradation of Organic Contaminants in Soils Treated With Quaternary Ammonium Cations

INTRODUCTION

Sorption of nonionic organic contaminants (NOCs) by soils is determined predominantly by the water solubility of the NOC and the soil organic matter content (8, 9, 16). Soil organic matter (SOM) is thought to act as an organic phase into which NOCs are solubilized (8, 9). This sorptive mechanism is commonly referred to as partitioning and is characterized by linear sorption isotherms over a wide solute concentration range, the lack of solute competition for sorption in multisolute systems, low and constant equilibrium heats of sorption, and an inverse dependence of sorption on the water solubility of the solute (8, 9). In high organic matter surface soils, contaminated by very poorly water soluble NOCs, sorption is high and leaching is minimal.

In contrast, subsoils and aquifer materials have inherently low organic matter contents and the migration of organic chemicals in these materials may ultimately result in groundwater contamination and human exposure. In the presence of water, mineral components of soils are deactivated as sorbents of NOCs due to the hydration of native inorganic cations exchanged on clays, or the preferential adsorption of water by other minerals such as silica or oxides. We have recently demonstrated that the sorption of NOCs by subsoils and aquifer materials can be greatly enhanced by treating them with organic cations of the form

14[$(CH_3)_3NR]^+$ or [$(CH_3)_2NRR']^+$, where R and R' are large (C-10 or greater) alkyl hydrocarbons (4, 5, 6, 14, 18). These cations have been shown to effectively displace naturally occurring inorganic metal cations on clay mineral surfaces and inner layers by simple ion-exchange reactions (14, 18, 25). The resultant formation of organic phases derived from the alkyl hydrocarbon moieties of the exchanged cations have high affinities for NOCs and facilitate their removal from water (4, 5, 6, 14, 18). Although they are mechanistically similar, the HDTMA-derived sorptive phase is about 10 to 30 times more effective than SOM (on a unit mass basis) for the removal of NOCs from water (4, 18).

It has been suggested that *in-situ* modification of subsoils and aquifer materials with hexadecyltrimethylammonium (HDTMA) or similar cations could create a sorptive zone (4, 6, 7, 18) to immobilize organic contaminants present in an advancing plume. Coupling enhanced contaminant immobilization with subsequent biodegradation of the immobilized contaminants would provide a comprehensive environmental restoration technology. One key aspect in our efforts to integrate the soil modification technology for immobilization of NOCs, with biodegradation, is the biocompatability of quaternary ammonium cations to soil and aquifer microorganisms. Quaternary ammonium cations such as HDTMA are known to be toxic to bacterial species while present in the dissolved form. *Streptococcus aureus* has a 0% survival rate at low uM concentrations of HDTMA (11). Cationic surfactants in streams receiving detergent-containing wastewater and in sewage sludges have toxic effects on the bacterial communities that are present (20, 23). Significant decreases in the growth rates of bacterioplankton and in the

photosynthetic rate of phytoplankton were observed at 0.03 to 0.1 mg/ml ditallowdimethylammonium chloride (DTDMAC) (23). Nonetheless, bacterial communities are able to adapt to relatively high concentrations of cationic surfactants when the concentration is raised slowly (21, 24). Furthermore, the strong adsorption of organic cations to soil and suspended particulates may substantially mitigate their toxicity (1, 10, 23) allowing for survival and continued activity of biodegradative bacterial populations.

This study evaluates the biocompatability of two quaternary ammonium cations, HDTMA and dimethyldioctadecylammonium bromide (DODMA), by measuring the biological mineralization of sorbed pollutants in HDTMA- and DODMA-modified soil and aquifer material.

MATERIALS AND METHODS

Chemicals- Radiolabeled substrates obtained from Sigma had a radiochemical purity >98% pure. These included [UL-^{14}C] d-glucose (8.7 mCi/mmol), [ring UL-^{14}C] toluene (51.5 mCi/mmol), [ring UL-^{14}C] 2,4-dichlorophenoxy acetate [2,4-D] (12.8 mCi/mmol), [ring UL-^{14}C] naphthalene (10.1 mCi/mmol), [ring UL-^{14}C] phenanthrene (13.1 mCi/mmol) and [ring UL-^{14}C] salicylate (7.6 mCi/mmol). [^{14}C]-HDTMA, labelled in the terminal (C-16) carbon was obtained from Moravek with a radiochemical purity of >97% and a specific activity of 55 mCi/mmol. HDTMA was obtained from Sigma (>99% purity) and DODMA was obtained from Kodak (>99%).

Soils- Two soil types were used in this study. The Marlette soil is a fine-loamy, mixed, mesic, glossoboric hapludalfs (Marlette series) collected from the Crop and Soil Sciences farm at Michigan State University. The second soil was collected from a shallow aquifer of approximately 7.5 feet in depth located in Alaska and will be referred to as the Eielson aquifer material. This soil had been previously contaminated with jet fuel. Soils were stored at 5C upon collection, then briefly air-dried and sieved through a 2-mm screen before use. Soils were characterized with respect to cation exchange capacity (CEC) by measuring the barium displacement of magnesium-saturated soils (19), organic carbon content by microcombustion (Huffman Laboratories, Golden,

Colo.), and particle size distribution by the hydrometer method (13).

Adsorption Isotherms- HDTMA adsorption isotherms were performed for each soil by the batch method. One gram of oven-dried soil (110C, 24 hours) was weighed into 25-ml glass Corex tubes equipped with teflon-lined screw caps. Solutions containing various concentrations of HDTMA (Sigma) were added to each tube. These solutions consisted of unlabeled HDTMA and sufficient [^{14}C]-HDTMA in phosphate buffered saline [PBS] (8.5g of NaCl, 0.3g of KH_2PO_4, 0.6g of Na_2HPO_4 per liter of distilled water, pH 7.0) to attain initial activities of approximately 5000 dpm ml^{-1}. A 1 ml portion of each solution was added to 7.5 ml of scintillation fluid to determine the initial activity. The soil-HDTMA suspensions were equilibrated overnight on a rotary shaker, centrifuged (9,000 x g, 25 min), and 1 ml aliquots of the surpernatants were withdrawn and added to 7.5 ml of scintillation fluid. The activity of [^{14}C]-HDTMA was determined by liquid scintillation counting (LSC) (Packard 1500 Tri-Carb Liquid Scintillation Analyzer). Counts were converted to disintegrations per minute by external standards quench correction, and the concentration of HDTMA in solution was determined. The concentration of HDTMA adsorbed to the soil was calculated by difference. Adsorption isotherms were constructed by plotting the concentration of adsorbed HDTMA (mmole/g) versus the concentration of HDTMA remaining in solution (mmole/ml).

Mineralization Assays- The heterotrophic potential of soil microbial populations to mineralize glucose, salicylate, 2,4-D, toluene,

naphthalene or phenanthrene following treatment with quaternary ammonium compounds was determined in modified Bartha-Pramer (3) flasks. These consisted of a 250 ml Erlenmeyer flask equipped with a teflon-lined screwcap and a teflon-capped, crimp-sealed sidearm reservoir. The conical sidearm reservoir containing 1 ml of 2N KOH (to trap evolved CO_2) was accessible via an 18 guage leur lock needle inserted through the septum and fitted with a short length of teflon tubing at the tip to allow quantitative recovery of alkali. These flasks represent an air-tight environment which may be sampled for $^{14}CO_2$ analysis without loss of substrate via volatilization.

Solutions of radiolabeled test substrates and/or cations were prepared in PBS and added to ten grams of briefly air-dried and sieved (2 mm) soil in flasks. The total activity was approximately 250,000 dpm per 50 ml to be added to each flask. One ml aliquots were removed to determine initial activities. Glucose (200 ug/ml), salicylate (5 ug/ml) and 2,4-D (10 ug/ml) solutions were amended with varying amounts of HDTMA or DODMA, corresponding to fixed percentages (0, 30, 50 and 70) of the soil cation exchange capacities (CEC). For naphthalene and phenanthrene, 100 ul of stock solutions in acetone was added directly to each of HDTMA or DODMA-containing PBS solution to give a final naphthalene and phenanthrene concentrations of 1 ug/ml and 0.5 ug/ml, respectively. [^{14}C]-Toluene was added to toluene at 2.5×10^6 dpm/ml and 230 ul of this solution was added to aliquots of PBS containing HDTMA or DODMA to give a final toluene concentration of 5 ug/ml. Each concentration of substrate and level of organic cation treatment was tested in duplicate flasks.

Soil slurries were incubated by shaking on a rotary shaker (200 rpm) at room temperature to maintain suspension of soil particulates and transfer of oxygen from the headspace. The 1 ml of potassium hydroxide solution was removed from the conical sidearm reservoir periodically, placed in 7.5 ml of liquid scintillation cocktail and replaced with 1 ml of fresh 2N KOH. After several hours in the dark to allow chemiluminescence to subside, samples were analyzed for radioactivity by liquid scintillation counting. Sterile controls were included by autoclaving the soil and biometer flasks three times at 15 psi and 250F for one hour on three consecutive days. [^{14}C]-HDTMA controls were included in which the mineralization of HDTMA (50% of soil CEC) was followed over time. For long-term experiments (greater than 1 month) air was periodically introduced into the flasks by loosening the screwcap and drawing air past the KOH solution by use of a 25 ml syringe. Results are plotted as the cumulative percentage of the added radioactivity recovered as $^{14}CO_2$ over time.

The influence of adding HDTMA in a prebound state to fresh soil on the mineralization of 2,4-D was also measured. Marlette B_t horizon soil was sterilized by autoclaving as described above. Sterile soil (100 g) was treated with HDTMA (in PBS) at 70% of the soil CEC. The treated soil was equilibrated by shaking on a rotary shaker overnight, centrifuging (7000 xg, 30 min) to remove unbound HDTMA, and air-drying the soil pellet. Adsorbed HDTMA concentrations were determined in duplicate samples prepared as above but spiked with [^{14}C]-HDTMA at 5000 dpm/ml. Following centrifugation, 1 ml of the supernatant was removed and counted to determine the HDTMA concentration. HDTMA-modified

smectite clay was prepared in a similar manner. The mineralization of ^{14}C-labelled 2,4-D (10 ug/ml) in Marlette A horizon soil was assayed following the addition of sterile HDTMA-treated Marlette B_t soil or HDTMA-treated smectite clay in an amount calculated to achieve the same HDTMA loading as treatments where free HDTMA was added to 10 g of Marlette A soil at 70% of the soil CEC.

Similarly, an assay was conducted to determine the effect of adding HDTMA prebound to soil on naphthalene mineralization. Ten grams of air dried and sieved (2mm) Bielson aquifer material was placed into biometer flasks and autoclaved as described previously. Aseptically, 50 ml of a PBS solution containing HDTMA (30% CEC) and naphthalene/^{14}C-naphthalene (2ppm and 250,000 dpm) was added to each biometer flask. Flasks were equilibrated by shaking (200 rpm) for 24 hours. To remove unbound HDTMA, 1 gram of sterilized Bielson aquifer material, 89 mg of sterile smectite clay, or 5 ml of a suspension of a pure culture of HDTMA-degrading bacteria (final density, 10^8 cells ml^{-1}) were added to duplicate biometer flasks. After three days, one gram of fresh Bielson aquifer material was added to each flask as a source of inoculum and mineralization of naphthalene was followed over time by measuring $^{14}CO_2$. Control flasks amended with unlabeled naphthalene and ^{14}C-HDTMA/HDTMA allowed the quantification of HDTMA mineralization in flasks receiving the pure culture of HDTMA-degrading bacteria.

Determination of Viable Numbers and Diversity- The effect of HDTMA on the viability and diversity of the aerobic soil bacterial community was examined. Ten grams of freshly collected, briefly air-dried Marlette A

horizon soil and Eielson aquifer material was weighed into sterile 250 ml Erlenmeyer flasks. Fifty ml PBS containing HDTMA in an amount equivalent to 50% of the respective soil CECs were added and the slurries were shaken at 200 rpm on a rotary shaker at 25C for one hour. Slurries were blended briefly in a Waring blender and diluted in PBS for plating onto various solid media in triplicate.

Dilutions were plated onto nutrient, PTYG, 1:20 PTYG and tap water agars. The nutrient agar contained 4 grams of nutrient broth (Difco) and 15 grams of agar (Difco) per liter of distilled water. PTYG agar plates contained 10 grams each of d-glucose (Baker) and yeast extract (Difco), 5 grams each of peptone (Sigma) and trypticase soy broth (BBL), 0.6 grams $MgSO_4.7H_2O$, 0.07 grams $CaCl_2.2H_2O$ and 15 grams of agar per liter of distilled water. The 1:20 PTYG agar media is a 1:20 dilution of the PTYG media, maintaining the agar at 15 grams per liter of distilled water. Tap water agar was prepared by adding 15 grams of agar to one liter of tap water. Each media type contained 100 milligrams per liter of cycloheximide (Sigma) to reduce fungal overgrowth on plates.

All plates were incubated at 25C in the dark for several weeks and evaluated with respect to colony morphology, color, size, and total numbers and diversity of colonies. Phenanthrene-degrading bacteria present in treated and untreated soil were enumerated by the agar overlay method (17). Dilutions of the soil slurries were plated onto nutrient agar and colonies were allowed to grow over a three week period. The agar plates were then sprayed with a phenanthrene solution (20 mg per ml DMSO). The solvent evaporated leaving a visible, crystalline film of phenanthrene. Active bacteria able to degrade

phenanthrene created clearing zones in this film and the absence of clearing zones indicated a lack of phenanthrene-utilizing bacteria.

RESULTS

The soils used in this study represented a range of cation exchange capacities and clay mineral contents as depicted in Table 1.1. HDTMA adsorption isotherms were obtained for the Marlette A and B_t horizon soils (Figure 1.1) and the Bielson aquifer material (Figure 1.2). In accordance with previous observations (5, 18) the cation exchange capacity (CEC) for each soil was approximated by the plateaus of the HDTMA adsorption isotherms (Figures 1.1 and 1.2). Adsorption isotherms were highly nonlinear, characterized by a steep initial rise in the isotherm followed by a plateau at or near the measured CECs of the soils. The aqueous phase concentration of HDTMA when added to a soil slurry at a given level could be predicted from these isotherms.

The effect of adding HDTMA at various loading rates (as a percentage of the estimated CEC) on the mineralization of several organic substrates was measured. Cumulative mineralization of glucose, salicylate and 2,4-D in the Marlette A horizon soil treated with HDTMA at 0% (control), 30%, 50% and 70% of the soil CEC is depicted in Figure 1.3. The curves show that with an increase in the amount of HDTMA added to the soil, the lag in the onset of mineralization of the substrate increases. Generally, the lag in response to mineralization of the substrate was a function of the amount of HDTMA added to the system and the complexity of the substrate (glucose < salicylate < 2,4-D) being

Table 1.1: Physical properties of the soils used in this study.

Soils	CEC[a]	%Clay	%Sand	%Silt	%OC[b]
Marlette A	11.5	9.4	54.9	35.7	2.2
Marlette Bt	12.8	17.2	51.5	31.3	0.6
Elelson	8.0	4.3	32.9	62.8	0.85

[a]Cation exchange capacity (milliequivalents/100 grams).
[b]Percent organic carbon (dry weight).

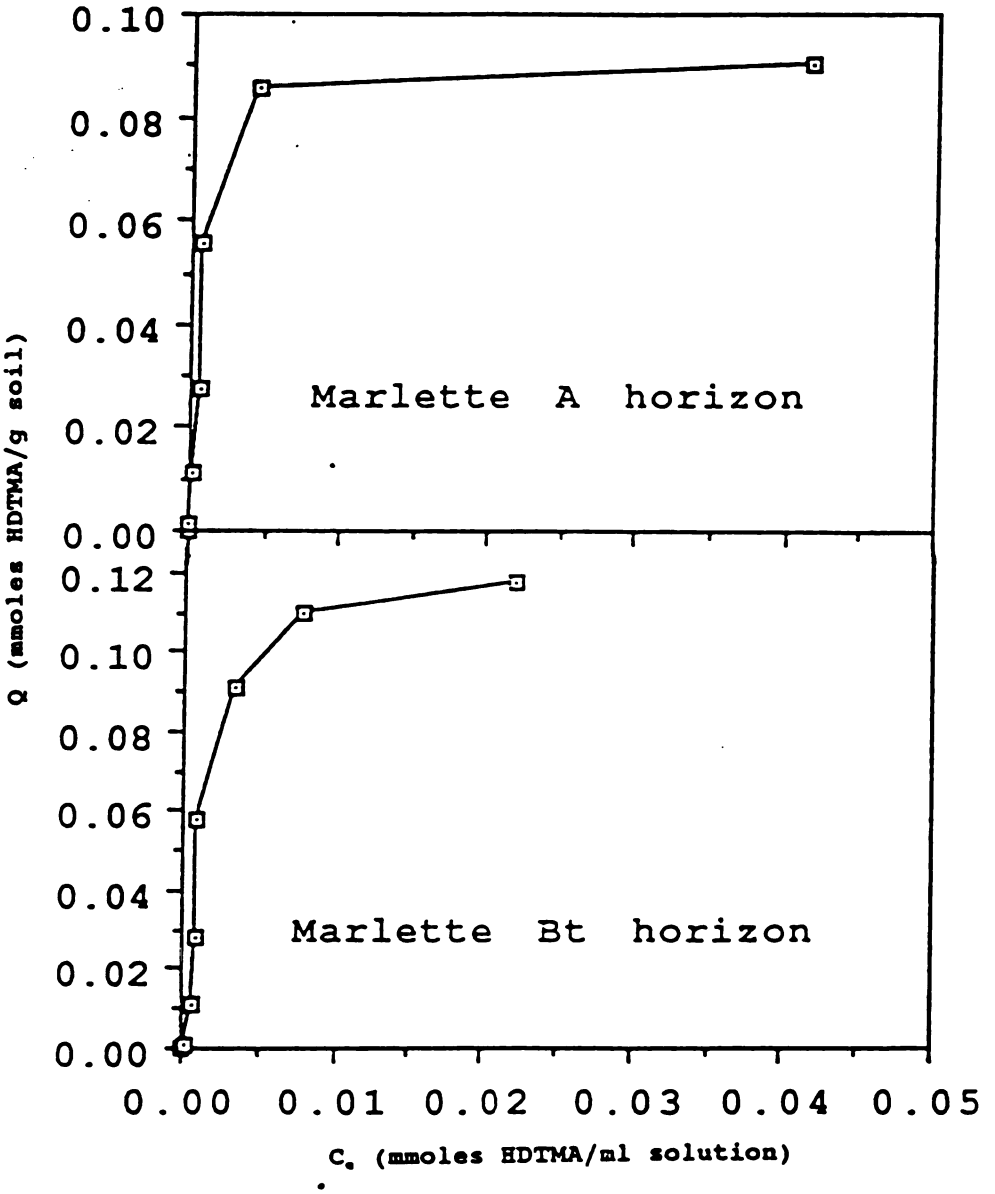

Figure 1.1: HDTMA sorption isotherms onto soils by the batch method.

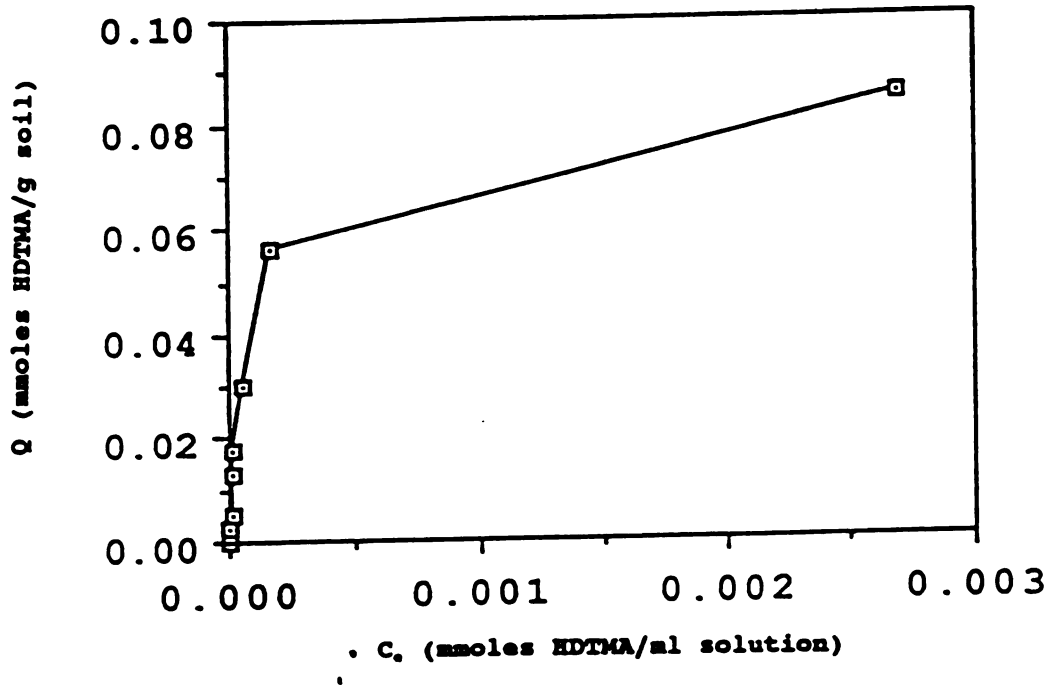

Figure 1.2: HDTMA sorption isotherms onto the Eielson aquifer material by the batch method.

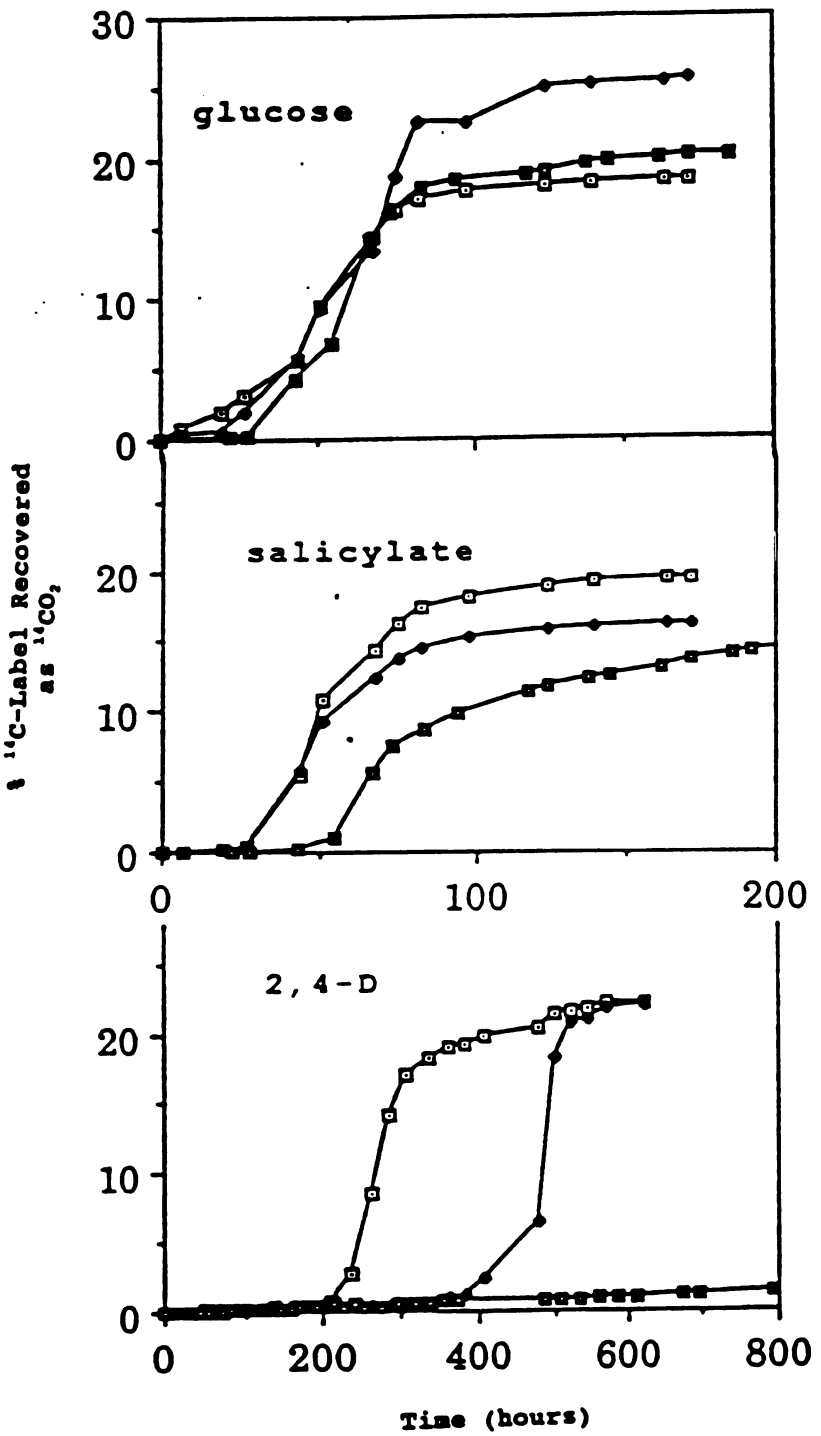

Figure 1.3: Mineralization curves in Marlette A horizon soil treated at 0% (no HDTMA, open boxes), 30% (125.8mg HDTMA, closed boxes) or 70% (293.5 mg HDTMA, closed diamonds) saturation of the soil CEC.

tested. Lag times were minimal in the case of glucose mineralization whereas initiation of 2,4-D mineralization had a long lag at the 30% CEC treatment level, nearly twice that of the control samples. Little or no mineralization activity was observed at the 70% CEC HDTMA treatment level, even after 800 hours. The lag times in mineralization of salicylate in the presence of HDTMA were intermediate, between that of glucose and 2,4-D; the lag times were similar (25h) between the control and 30% CEC HDTMA treatment and approximately doubled for the 70% CEC HDTMA treatment.

The extents of mineralization for each type of substrate was not strongly affected by the addition of HDTMA at any level, except for the 70% CEC HDTMA treatment for 2,4-D in which there was little $^{14}CO_2$ evolved during an 800 hour incubation. In some of the samples, such as the 30% CEC HDTMA treatment with glucose as a substrate, the extent of mineralization actually increased as compared to the control.

Mineralization assays for glucose, toluene, naphthalene and phenanthrene were also conducted utilizing the Eielson aquifer material (Figures 1.4 and 1.5). The Eielson aquifer material was treated at 0%, 10%, 30% and 70% of its CEC. Generally, the more HDTMA added to the soil, the longer the lag time prior to the onset of mineralization of the substrate, as was observed with the Marlette soil. Although the degradative capacity for glucose, toluene and naphthalene was preserved in the HDTMA-treated soils, the extents of substrate mineralization were affected by the presence of HDTMA in the Eielson soil, with treated samples evolving a lower percentage of the substrate as $^{14}CO_2$. The addition of HDTMA at 70% of the CEC resulted in a reduction in the

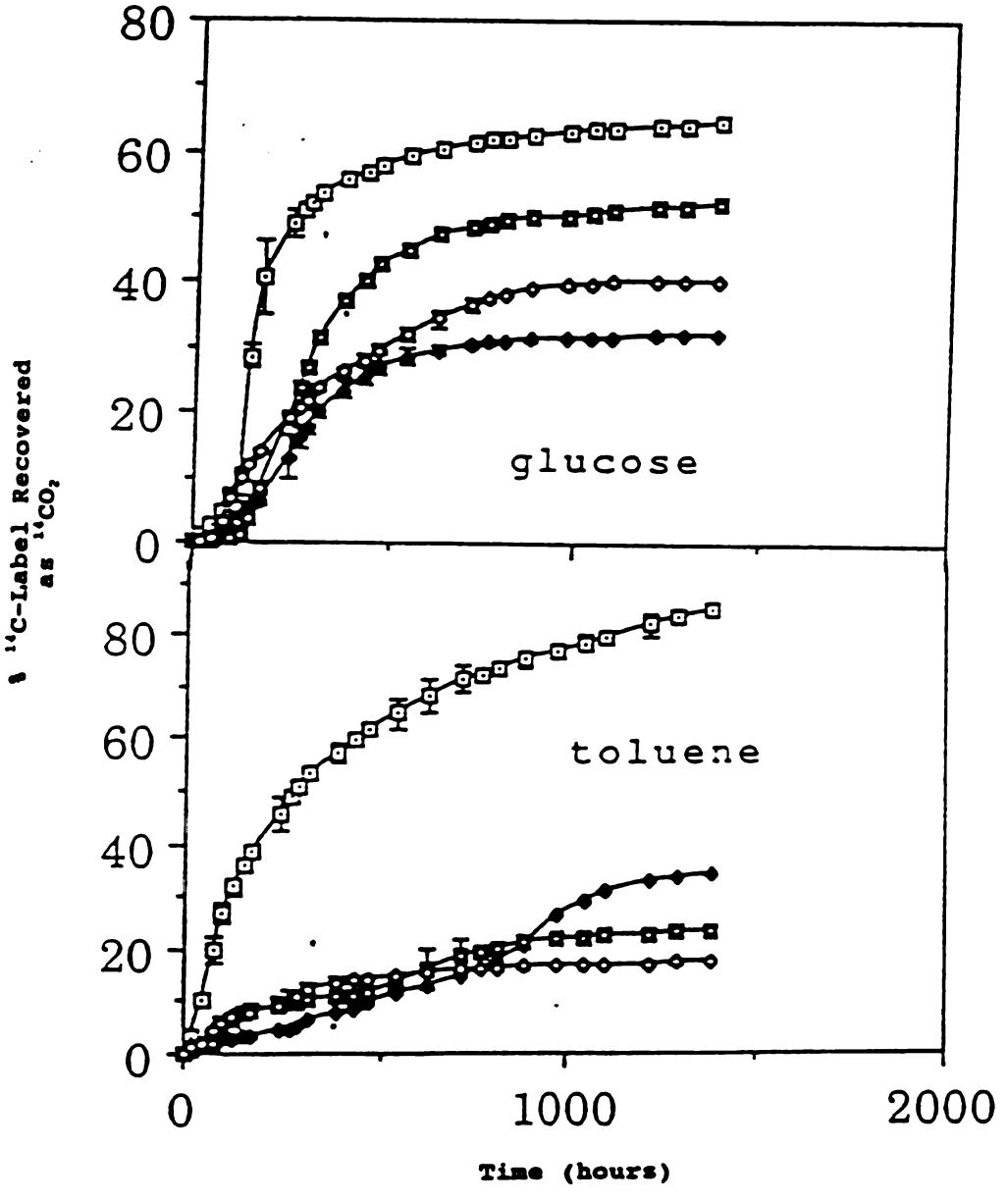

Figure 1.4: Mineralization curves in the Eielson aquifer material treated at 0% (no HDTMA, open boxes), 10% (29.2 mg HDTMA, closed boxes), 30% (87.5 mg HDTMA, open diamonds) or 70% (204.2 mg HDTMA, closed diamonds) saturation of the soil CEC.

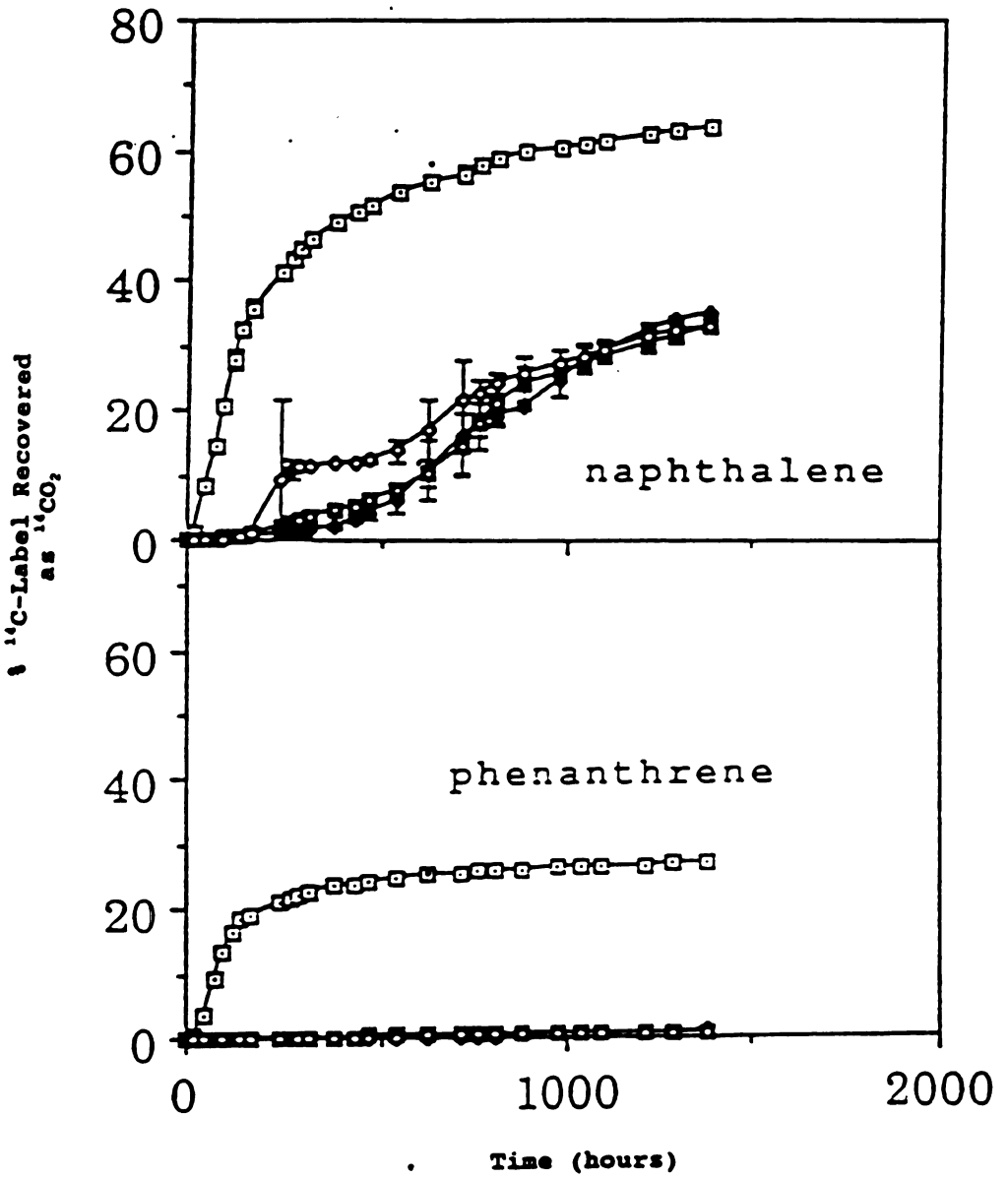

Figure 1.5: Mineralization curves in the Eielson aquifer material treated at 0% (no HDTMA, open boxes), 10% (29.2 mg HDTMA, closed boxes), 30% (87.5 mg HDTMA, open diamonds) or 70% (204.2 mg HDTMA, closed diamonds) saturation of the soil CEC.

extent of mineralization of about one half.

Phenanthrene mineralization in the Eielson soil was completely inhibited by HDTMA at all treatment levels. To determine whether this was due to lowered availability (i.e. increased sorption) resulting from HDTMA-treatment, or to the lack of degradative organisms, phenanthrene degrading organisms were enumerated. Phenanthrene degrading microorganisms of several types were present in high numbers (>10^4 per ml) in the untreated Eielson aquifer material but were absent in the samples treated with HDTMA.

In the above experiments, HDTMA was introduced to the soil as an aqueous solution, i.e. initially present as the free, unbound cation. Two experiments were conducted to determine whether the toxic effects of HDTMA can be alleviated when it is prebound to soil components prior to its exposure to degrading microorganisms in soil. As previously shown, 2,4-D is not mineralized in the Marlette A horizon soil when treated with HDTMA at 70% of the CEC (Figure 1.3). Figure 1.6 shows the mineralization curves for 2,4-D in the Marlette A horizon soil when an equivalent amount of HDTMA, prebound to Marlette B_t horizon soil or smectite clay, was added. These curves show that 2,4-D is not mineralized when HDTMA is introduced as the free cation at 70% of the CEC. However, when an equivalent amount of HDTMA is prebound to smectite clay or soil, 2,4-D is readily mineralized.

Similar results were obtained from an experiment conducted using the Eielson aquifer material. HDTMA at 50% of the soil CEC and naphthalene were added together in PBS to sterile soil and allowed to equilibrate. To remove soluble or unbound HDTMA clay, sterile soil or

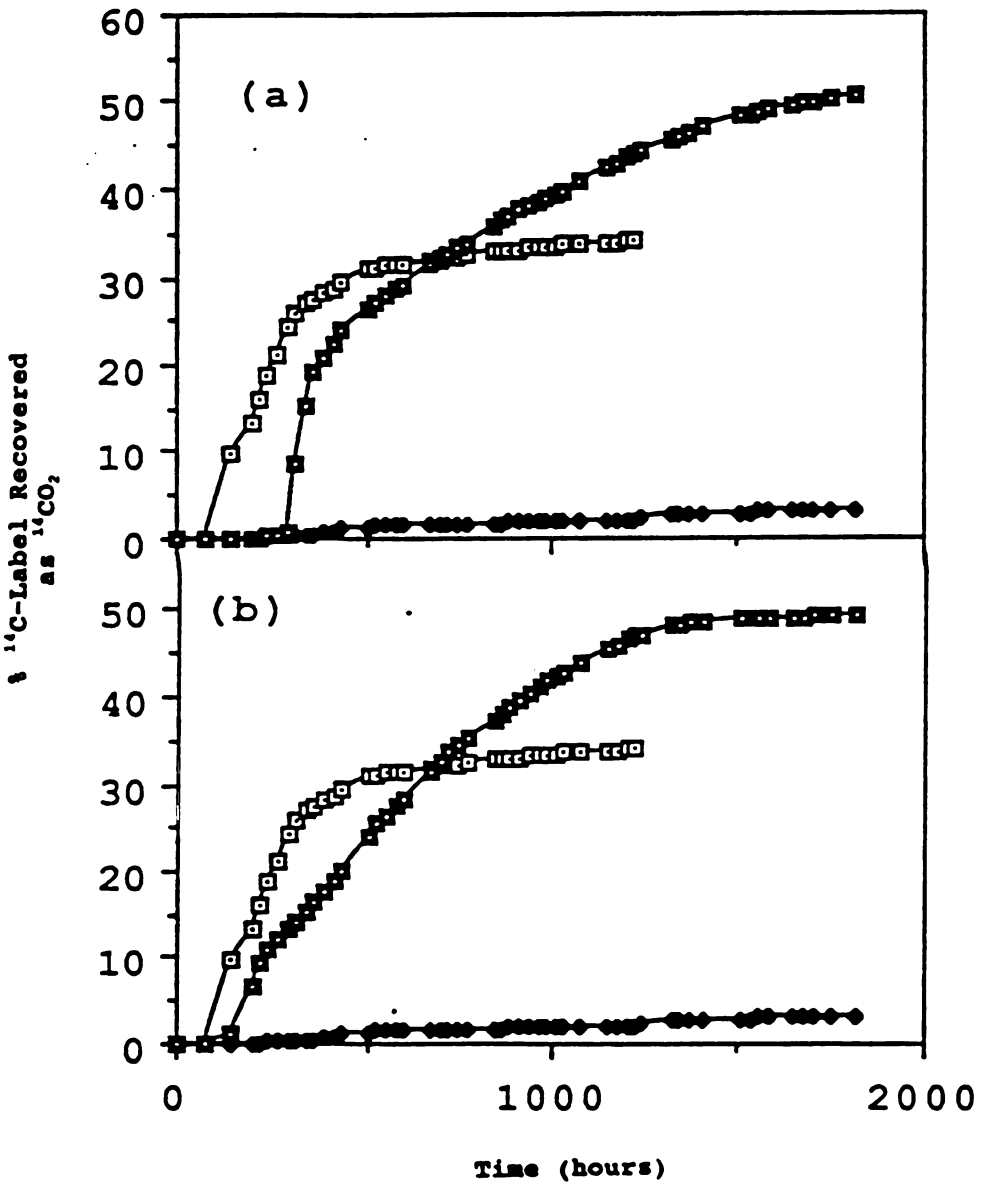

Figure 1.6: Mineralization curves for 2,4-D in Marlette A horizon soil when HDTMA is added as the free cation in an amount to saturate the CEC at 0% (no HDTMA, open boxes) or 70% (293.5 mg HDTMA, closed boxes) or an equivalent amount of HDTMA to accomplish 70% saturation prebound to clay(a) or soil(b).

known HDTMA-degrading microorganisms were added to the soil slurries. After a second equilibration period, flasks were inoculated with one gram of fresh aquifer material serving as the source of inoculum was added and naphthalene mineralization monitored. The mineralization curves are represented in Figure 1.7, and show that the lag in response to mineralization of naphthalene in the presence of HDTMA can be decreased by additions of clay or HDTMA degrading bacteria. The extent of mineralization of naphthalene was also increased as compared to the HDTMA-treated control for all of the additions. Incubations with soil treated with ^{14}C-labeled HDTMA showed that over the time of the second equilibration period (50 hours) that 36% of the ^{14}C label was recovered as $^{14}CO_2$ when the HDTMA-degrading bacteria were added, while no $^{14}CO_2$ was recovered from the other treatments.

A second quaternary ammonium cation, DODMA, was evaluated for its effect on the mineralization of salicylate and naphthalene in the Eielson aquifer material. DODMA differs from HDTMA by having two large (C-18) alkyl hydrocarbon moieties where HDTMA has only a single (C-16) alkyl hydrocarbon moiety. DODMA was added in amounts equivalent to 0% (control) and 50% saturation of the CEC of the Eielson aquifer material. Figure 1.8 displays the cumulative mineralization of each substrate. In these experiments, neither the lag time nor the extent of mineralization was substantially affected by the addition of DODMA at 50% of the CEC.

Table 1.2 shows the effect that HDTMA has on the diversity and viable counts of aerobic bacteria in each soil. A large reduction in diversity and numbers of the bacterial community can be seen after a one hour exposure to HDTMA at 50% of the cation exchange capacity of the

Figure 1.7: Mineralization curves for naphthalene in Eielson aquifer material when HDTMA at 50% saturation of the CEC was added to sterile soil. After an equilibration period (24h), additions of 1 g of sterile aquifer material (closed boxes with dot), 88.9 mg smectite clay (open diamonds), 10^8 cells/ml of a known HDTMA degrader (closed boxes), or no addition control (closed diamonds) before 1 g of fresh aquifer material was added as a source of inoculum and naphthalene mineralization followed. A no HDTMA control was also included (open boxes).

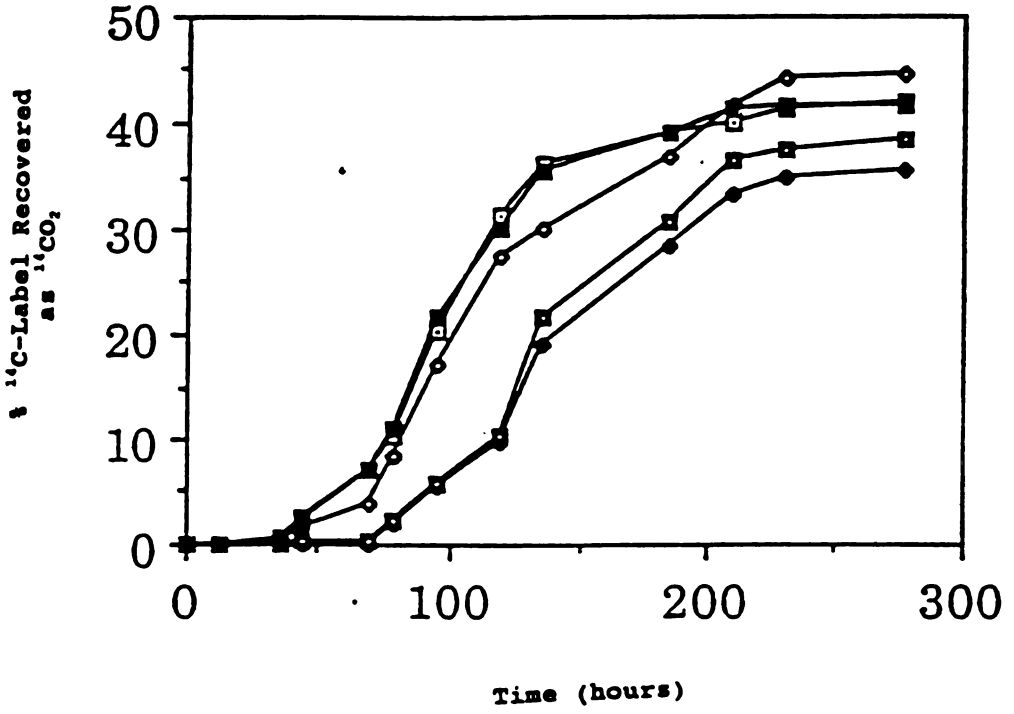
% ^{14}C-Label Recovered as $^{14}CO_2$
50
40
30
20
10
0
0
100
200
300
Time (hours)

Figure 1.7:

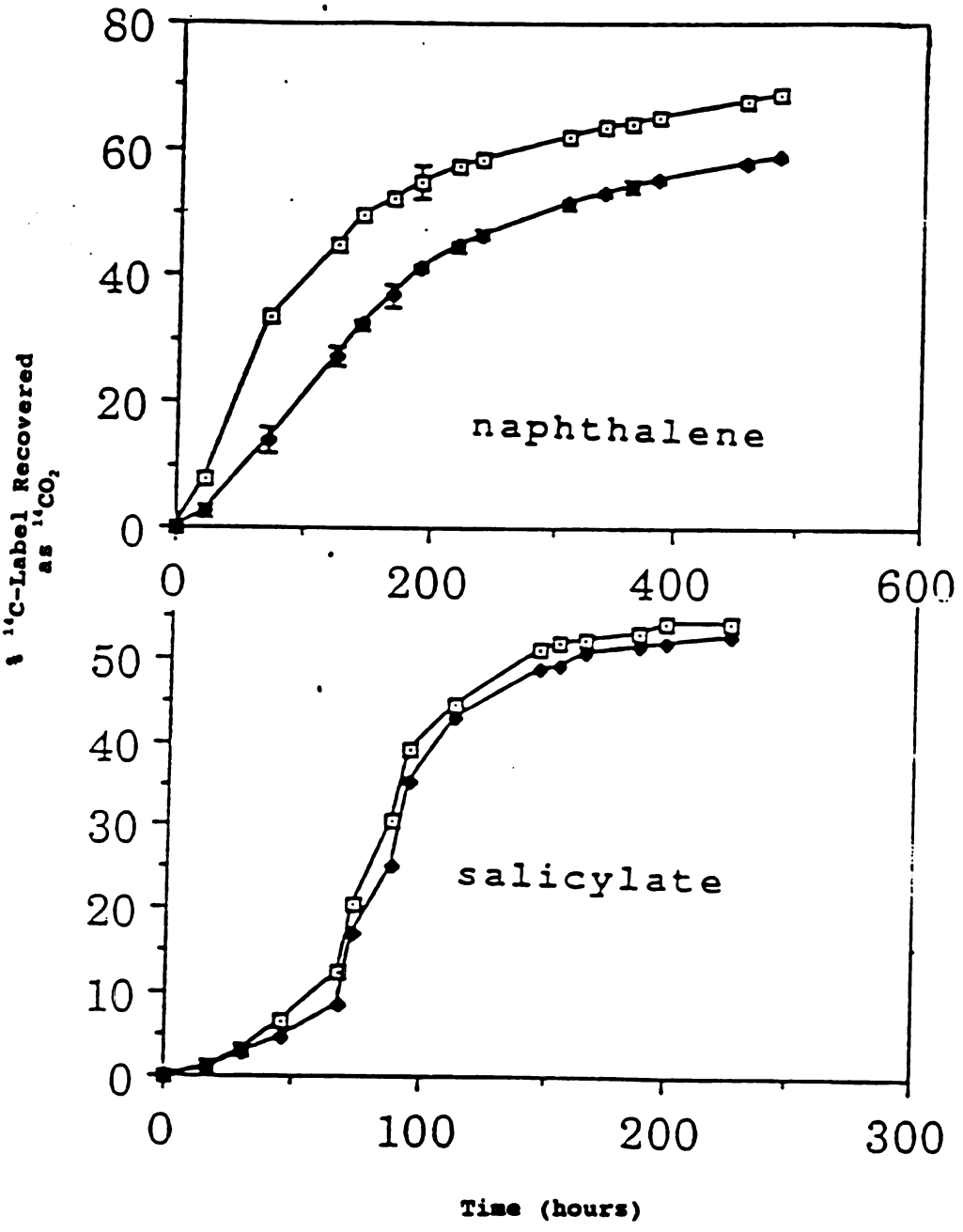

Figure 1.3: Mineralization curves in the Bielson aquifer material treated at 0% (no DODMA, open boxes) or 50% (252.4 mg DODMA, closed boxes) saturation of the soil CEC.

Table 1.2: Microbial numbers and diversity in soils untreated and treated with HDTMA.

Soil	Media[a]	Treatment[b]	# Colony Types	# Bacteria[c]
Elelson	1	control	14	$7.73x10^5$
	1	HDTMA	4	$4.00x10^4$
	2	control	12	$2.86x10^5$
	2	HDTMA	5	$1.66x10^4$
	3	control	17	$9.73x10^5$
	3	HDTMA	7	$6.73x10^4$
	4	control	5	$5.46x10^5$
	4	HDTMA	2	$5.21x10^4$
Marlette	1	control	18	$2.09x10^6$
	1	HDTMA	9	$5.27x10^5$
	2	control	14	$9.39x10^5$
	2	HDTMA	6	$5.91x10^4$
	3	control	20	$3.14x10^6$
	3	HDTMA	9	$7.21x10^5$
	4	control	5	$6.09x10^5$
	4	HDTMA	3	$5.16x10^4$

[a]Media 1 corresponds to Nutrient, media 2 to PTYG, media 3 to 1:20 PTYG and media 4 to Tap Water.
[b]Controls were incubated in PBS, HDTMA treatments at 50% saturation of the CEC, each for one hour prior to plating.
[c]Represents the number of viable bacteria per gram of soil, dry weight.

soil. Generally, a one log reduction in numbers can be seen upon treatment with HDTMA. Diversity of the bacterial community is reduced from 12 to 16 bacterial types in the no treatment controls to only a few types present in the HDTMA treated samples.

DISCUSSION

In this study we have attempted to assess the toxicity of quaternary ammonium cations to bacterial communities in soils and subsoils as reflected by the mineralization of common organic contaminants, and the overall diversity and size of the aerobic, heterotrophic soil bacterial community. In this way, the feasibility of a comprehensive soil restoration technology utilizing quaternary ammonium cations such as HDTMA and DODMA to reduce transport of organic contaminants in a modified soil or aquifer material, followed by subsequent biodegradation of the immobilized contaminants, may be assessed.

The chief objective of this study was to determine if biodegradative capacity of soil microorganisms for common organic contaminants and the pesticide 2,4-D would be maintained upon modification of the soil with quaternary ammonium cations. We have found that when HDTMA is introduced to a soil-water system in the free cationic form an initial toxic effect is evident as indicated by the lag time before mineralization of added substrates commences. The onset of mineralization was delayed by the addition of HDTMA, with the magnitude of the lag directly related to the level of HDTMA treatment, expressed as a percent of the soil cation exchange capacity (CEC). This is best shown in the mineralization curves for glucose, salicylate and 2,4-D (Figure 1.3).

We also observed that with an increase in the substrate complexity (glucose < salicylate < 2,4-D) the magnitude of the lag increased. This can be explained by considering the size of the bacterial population able to utilize a more complex substrate. Additions of HDTMA to soil removes a certain percentage of the community, resulting in lower numbers of microorganisms able to degrade a given substrate, or totally eliminating them. If the size and/or diversity of the bacterial population(s) able to degrade 2,4-D, for instance, is smaller than the population able to utilize glucose, the chance that 2,4-D utilizers will be eliminated from the viable population by HDTMA treatment increases. If a large percentage of the 2,4-D utilizing bacteria are killed, the time required for repopulation to a level where a significant amount of 2,4-D degradation may occur will be lengthened. This is evident in the mineralization assays conducted in the Marlette soil and Eielson aquifer material (Figures 1.3, 1.4 and 1.5). Mineralization of naphthalene in the Eielson material had a longer lag than glucose and toluene, and phenanthrene biodegradation was totally eliminated at all HDTMA treatment levels.

The results in the Eielson aquifer material may be somewhat confounded by the phenomena of bioavailability of the nonionic substrates. By increasing the sorption coefficient of the soil or aquifer material with additions of HDTMA (4, 18), the aqueous phase concentration of a compound such as naphthalene and phenanthrene will be lowered possibly resulting in lowered mineralization rates. This could account for the decreased rates of mineralization of phenanthrene, which was not mineralized at the 30%, 50% and 70% CEC treatment levels,

because it has the lowest water solubility of the compounds tested and hence greatest sorption coefficient in the HDTMA-treated soils. As a result, it should be most susceptible to reduced bioavailability. Examination for the presence of phenanthrene degraders in the treated soil showed no degraders present whereas in the non-treated soil phenanthrene degraders were present. This indicates that the lack of degradation is primarily due to the elimination of the active population.

The extent of mineralization of glucose was actually increased with HDTMA treatment in the Marlette soil. The increased evolution of carbon dioxide from glucose respiration may be explained by considering the treatment with HDTMA as a fumigation process (15). Bacterial survivors may be able to utilize killed cells as an energy source and increase the size of their population, resulting in higher metabolic activities. The lowered extent of mineralization found for other substrates such as naphthalene upon treatment of the soil with HDTMA may result from different bacterial populations being dominant in the HDTMA treated soil as compared to that of the control.

HDTMA, once applied to a soil, will predominantly be bound to cation exchange sites of mineral components and organic matter. The affect of HDTMA when added to a soil in a prebound state, either to smectite clay or Marlette B_t soil, was investigated. When HDTMA was added in the prebound form at 70% of the soil CEC, 2,4-D was fully mineralized. However, when added at 70% saturation of the CEC as the free cation, 2,4-D was not mineralized. These results indicate that the lack of 2,4-D mineralization when HDTMA is added as the free cation is

the result of an initial toxicity and not reduced bioavailability. Once bound to the soil components, HDTMA is relatively nontoxic to degrading microorganisms.

The effect of prebound HDTMA on mineralization of naphthalene was similar. HDTMA was added to sterile soil at 50% of the CEC followed by additions of smectite clay, sterile soil or a known HDTMA degrader. These additions were made in an attempt to bind any residual free HDTMA or allow for the biodegradation of free HDTMA. Fresh soil was then added as a source of inoculum and mineralization of naphthalene was followed. The extents of mineralization for each treatment were elevated as compared to the HDTMA treatment without additions. The addition of an HDTMA degrading microorganism resulted in evolution of 36% of the radiolabeled HDTMA, which indicates that the lack of toxicity to the naphthalene utilizing bacteria was a result of the lowered level of residual free HDTMA present in the system. The lowered toxicity upon addition of soil or smectite clay is likely the result of a lower aqueous phase concentration of the HDTMA cation due to increased adsorption of otherwise unbound HDTMA. These results suggest that the presence of additional cation exchange capacity in a natural setting should result in lower levels of unbound HDTMA and hence less toxic effects of added HDTMA.

The effect DODMA has on the mineralization of salicylate and naphthalene in the Bielson aquifer material was investigated. It was found that DODMA had little or no effect on mineralization of these compounds. In related studies using pure cultures of soil bacteria, DODMA has been shown to be characteristically less toxic than HDTMA

(data not shown). This may be due to conformational or size restrictions not allowing for interactions of DODMA with cell surfaces. These phenomena will be investigated further as it pertains to the soil modification technology.

This study indicates that the initial treatment of a soil system with HDTMA in amounts up to 70% of the soil CEC has a detrimental impact on the microbial population and subsequent mineralization of common organic contaminants (toluene, naphthalene, phenanthrene) and 2,4-D, but once bound to soil components HDTMA is rendered relatively nontoxic to bacterial degraders. Similar treatment of aquifer material with DODMA appears to have little if any detrimental effect on the biodegradation potential for naphthalene. Treatment of soil with quaternary ammonium cations initially may kill a percentage of the bacterial community, but the relative non-toxicity of bound cations to bacterial degraders suggests the likelihood that the treated sorptive zone could be repopulated by bacteria associated with the flow of groundwater or those moving into the treated zone from adjacent nontreated areas. This study indicates that the soil modification utilizing organic cations to enhance sorption and reduce transport of contaminants, coupled with subsequent biodegradation of sorbed pollutants is a feasible and potentially useful soil remediation approach.

REFERENCES

1. Aronstein, Boris N., Yolanda M. Calvillo and Martin Alexander. 1991. Effect of surfactants at low concentrations on the desorption and biodegradation of sorbed aromatic compounds in soils. Environ. Sci. Technol. 25:1728-1731.

2. Beaubien, A., L. Keita and C. Jolicoeur. 1987. Flow microcalorimetry investigation of the influence of surfactants on a heterogeneous aerobic culture. Appl. Micro. Ecol. 53:2567-2573.

3. Bartha, R. and D. Pramer. 1965. Features of a flask and method fore measuring persistence and biological effects of pesticides in soil. Soil Science. 409:68-70.

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

5. Boyd, S.A., M.M. Mortland and C.T. Chiou. 1988b. Sorption characteristics or organic compounds on hexadecyltrimethylammonium smectite. Soil Sci. Soc. Amer. J. 52:652-657.

6. Boyd, S.A., WF Jaynes and BS 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 in Sediments in Water, Vol. 1. Lewis Publishers, Chelsea, MI.

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

8. Chiou, C.T., L.J. Peters 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. 18:295-297.

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

11. Gilbert, P. and A. Al-taae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Lett. Appl. Microbiol. 1:101-104.

12. Gordon, A.S. and F.J. Millero. 1985. Adsorption mediated decrease in the biodegradation rate of organic compounds. Microb. Ecol. 11:289-298.

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

14. Jaynes, W.F. and S.A. Boyd. 1990. Clay mineral type and organic compound sorption by hexadecyltrimethylammonium-exchanged clays. Soil

Sci. Soc. Amer. J.

15. Jenkinson, D.S. 1966. Studies on the decomposition of plant material in soil. II. Partial sterilization of soil and the soil biomass. J.Soil Sci. 17:280-302.

16. Karickhoff, S.W., D.S. Brown and T.A. Scott. 1979. Sorption of hydrophobic pollutants on natural sediments. Water Res. 13:241-248.

17. Kiyohara, H., K. Nagao, and K. Yano. 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. AEM 43:454-457.

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

19. Rhoades, J.D. 1982. Cation Exchange Capacity, p.149-157. *In* A.L. Page, R.H. Miller, and D.R. Keeney(ed.), Method of soil analysis, part 2. American Society of Agronomy, Madison, Wis.

20. Shabtai, Y. and D. Gutnick. 1985. Tolerance of Acinetobacter calcoaceticus RAG-1 to the cationic surfactant cetyltrimethylammonium bromide: role of the bioemulsifier emulsan. Appl. Environ. Microbiol. 49:192-197.

21. Shimp, Robert J., Burney S. Schwab and Robert J. Larson. 1989. Adaptation to a quaternary ammonium surfactant by suspended microbial communities in a model stream. Environ. Tox. and Chem. 8:723-730.

22. Song, H.G. and R. Bartha. 1990. Effects of jet fuel spills on the microbial community of soil. Appl. Environ. Microbiol. 56:646-651.

23. Tubbing, Diny M.J., and Wim Admiraal. Dec. 1991. Inhibition of bacteria and phytoplanktonic metabolic activity in the lower river Rhine by ditallowdimethylammonium. Appl. Environ. Microbiol. pp. 3616-3622.

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

25. Xu, S. and S.A. Boyd. 1992. Soil modification for enhanced removal of organic contaminants from aqueous solutions: Hexadecyltrimethylammonium (HDTMA)-soil interactions. Agronomy Abstracts.

Toxicity of Quaternary Ammonium Cations To Soil Microorganisms

INTRODUCTION

The sorption and transport of nonionic organic contaminants (NOCs) in soils is determined predominantly by the water solubility of the NOC and the soil organic matter content (8, 9, 16, 31). In the presence of water, mineral components of soil are deactivated as sorbents of NOCs due to the hydration of native inorganic cations exchanged on clays, or the preferential adsorption of water by other minerals such as silica or oxides. Soil organic matter (SOM) is thought to act as a partition phase into which NOCs are solubilized (8, 9, 16, 31). The extent of sorption is determined by the relative solubilities of NOCs in SOM and water. One important manifestation of this sorptive mechanism, commonly referred to as partitioning, is the high mobility of NOCs with relatively high water solubilities (e.g. benzene, trichloroethene) in low organic matter surface soils, subsoils and aquifer materials.

We have recently shown that the sorptive properties of such soils and subsoils for NOCs can be substantially enhanced by the addition of quaternary ammonium cations (QACs) of the form $[(CH_3)_3NR]^+$ and $[(CH_3)_2NRR']^+$, where R and R' are large (>C-10) alkyl hydrocarbon groups (4, 5, 18). Addition of QACs such as hexadecyltrimethylammonium (HDTMA) to soil results in the formation of an organo-clay complex through ion exchange reactions (5). These reactions displace naturally occurring

inorganic metal cations such as Ca^{2+} and Mg^{2+} from the cation exchange sites of soil clays. Sorption coefficients for common groundwater contaminants such as benzene increased by approximately two orders of magnitude in HDTMA-treated B horizon soils (4, 5, 18). The increased sorptive capacity of HDTMA-modified soils is due to the formation of an effective partition phase derived from the alkyl hydrocarbon moieties of HDTMA. This partition phase, fixed on the surfaces and interlayers of clays, functionally and compositionally resembles a bulk hydrocarbon solvent phase. The log K_{om} values for HDTMA-treated subsoils were approximately equal to the corresponding log K_{ow} values, and 10 to 30 times greater than K_{om} values obtained for untreated surface soils (i.e. natural soil organic matter). The demonstrated ability to substantially enhance the sorption of organic contaminants via this soil modification technology, may effectively decrease the transport of contaminants in the subsurface (6, 19). Coupling this with subsequent bioremediation of the immobilized contaminants offers the potential of a new, comprehensive *in situ* soil restoration technology.

Quaternary ammonium cations, such as HDTMA, are an important class of cationic surfactants commonly found in detergents, fabric softeners, disinfectants and hair conditioners. U.S. consumption of alkyldimethylbenzylammonium quaternary ammonium compounds, representing only one type of quaternary ammonium cation, was estimated to be 20-25 million pounds in 1979 (3). Due to the numerous uses and large consumption of these types of chemicals, they are frequently released to surface waters and wastewater streams. Previous studies have emphasized the fate and toxicity of quaternary ammonium cations in aquatic environments with

comparatively little focus on toxicity to bacteria (1, 2, 3, 7, 12, 20, 27).

Quaternary ammonium cations are toxic to bacteria at concentrations commonly in the low uM range, with gram positive bacteria being somewhat more resistant than gram negative bacteria (12). QACs released to streams and rivers have been shown to be toxic to bacteria and algae with a dependance on the concentration of suspended material present in the waterway (27). Adsorption of the QAC to the suspended matter has been implicated as the likely factor in reducing toxicity. It is well established that organic cationic compounds interact strongly with soils concomitantly reducing their bioavailability to microbial degraders (10, 21, 29, 30). Studies have also shown that as the length of the QAC alkyl chain is increased, the resultant toxicity to bacteria is increased (12). For instance, an increase of the alkyl chain length of a trimethylalkylammonium molecule from C-9 to C-12 to C-16 results in increased toxicity to bacterial species.

Although quaternary ammonium cations have been shown to be generally toxic to bacteria, differential responses to organic cations among bacterial types as well as resistant populations have been observed (11, 12, 17, 23). A bacterium capable of survival in relatively high concentrations of HDTMA has been studied (25). A plasmid encoded protein residing in the bacterial membrane was shown to afford resistance to HDTMA for the bacterium. It has also been shown that various bacterial types have differing reactions to HDTMA, indicating each cell type may have different mechanisms for HDTMA interactions (12). Furthermore, studies have indicated that bacterial

populations are able to adapt to relatively high concentrations of quaternary ammonium cations given an adjustment period and slow rise in concentration. This adaptation is lost when quaternary ammonium cations are removed for a period of time (23).

The overall goal of this study is to determine if soil modification with QACs (e.g. HDTMA) to increase immobilization of organic contaminants can be integrated with bioremediation of the sorbed pollutants. One key issue in the development of this technology is the bactericidal properties of HDTMA or similar QACs to microbial pollutant degraders present in soils. In this study we measured the effect HDTMA has on the size and diversity of the soil heterotrophic bacterial population, and examined the characteristics of the bacteria able to survive modification of the soil with HDTMA. We also measured the toxicity of various quaternary ammonium cations to a single bacterial strain, and the toxicity of HDTMA to fifteen bacterial types. We attempted to correllate HDTMA toxicity with the distribution coefficient of HDTMA to each bacterial type, as well as the cellular hydrophobicity of each strain. The rate at which HDTMA exerts toxicity to a single bacterial strain was evaluated. Also, the alleviation of HDTMA toxicity to bacteria by adding smectite clay to adsorb aqueous phase HDTMA was examined.

MATERIALS AND METHODS

Soil- A freshly collected surface soil treated with HDTMA was our source of bacterial isolates for characterization and use in further studies. The soil was classified as a fine-loamy, mixed, mesic, glossoboric hapludalfs (Marlette series) collected from the Crop and Soil Sciences farm at Michigan State University. An Alaskan aquifer material previously contaminanted with jet fuel, designated Eielson aquifer material, was included in studies on the effect of HDTMA on bacterial population size and diversity. The soils were analyzed for particle size distribution by the hydrometer method (13), organic carbon content by microcombustion (Huffman Laboratories, Golden, CO) and cation exchange capacity (CEC) by measuring the barium displacement of magnesium-saturated soil (22). The Marlette soil was collected in May, less than 24 hours before use in isolation procedures. Both soils were briefly air dried and sieved through a 2 mm screen.

The Marlette A horizon soil had an organic carbon content of 2.2% and contained 9.4% clay, 54.9% sand and 35.7% silt. The CEC was 11.5 milliequivalents per 100 grams of soil. The Eielson aquifer material contained 0.85% organic carbon, 4.3% clay, 32.9% sand and 62.8% silt. It had a CEC of 8.0 milliequivalents per 100 grams of soil.

HDTMA Adsorption Isotherms- Hexadecyltrimethylammonium (HDTMA) adsorption isotherms were performed for the Marlette A horizon soil and

the Bielson aquifer material by the batch method. One gram of oven-dried soil (110C, 24 hours) was weighed into 25 ml glass Corex tubes equipped with teflon-lined screw caps. Solutions containing various concentrations of HDTMA (Sigma) were added to each tube. These solutions consisted of unlabeled HDTMA and sufficient [^{14}C]-HDTMA (>98% radiochemical purity, 55mCi/mmol, Moravek) in phosphate buffered saline [PBS] (8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of Na_2HPO_4 per liter of distilled water, pH 7.0) to attain initial activities of approximately 5000 dpm ml^{-1}. A 1 ml portion of each solution was added to 7.5 ml of scintillation fluid to determine the initial activity. The soil-HDTMA suspensions were equilibrated overnight on a rotary shaker, centrifuged (9,000 x g, 25 min.) and 1 ml aliquots of the surpernatants were withdrawn and added to 7.5 ml of scintillation fluid. The [^{14}C]-HDTMA activity was determined by liquid scintillation counting (LSC) (Packard 1500 Tri-Carb Liquid Scintillation Analyzer). Counts were converted to disintegrations per minute by external standards quench correction, and the concentration of HDTMA in solution was determined. The concentration of HDTMA adsorbed to the soil was determined by difference. Adsorption isotherms were constructed by plotting the concentration of adsorbed HDTMA (mmole/g) versus the concentration of HDTMA remaining in solution (mmole/ml).

Isolation Procedure- Isolation of bacteria from the Marlette A horizon soil for characterization, enumeration and use in further experimentation, and enumeration of bacteria in the Bielson aquifer material, was carried out by treating ten grams of the freshly

collected, sieved soils at 50% saturation of the soil CEC with HDTMA in 50 ml of PBS solution. After a one hour incubation, the soil slurry was blended in a Waring blender for three minutes. The blended slurry was further diluted in PBS before plating onto four solid media types including nutrient, PTYG, 1:20 PTYG and tap water. Nutrient agar plates consisted of 4 grams nutrient broth (Difco) and 15 grams of agar (Difco) per liter of distilled water. PTYG media contained 10 grams each of glucose and yeast extract, 5 grams each of trypticase soy broth and peptone, 0.6 grams $MgSO_4.7H_2O$, 0.07 grams $CaCl_2.2H_2O$ and 15 grams of agar per liter of distilled water. The 1:20 PTYG media is a 1:20 dilution of the PTYG media, maintaining the agar concentration at 15 grams per liter. The tap water agar plates consisted of 15 grams of agar per liter of distilled water. Each media type contained cycloheximide (Sigma) at 100 mg/L to reduce fungal overgrowth. Plates were incubated in the dark at 25C for several weeks. Bacterial colonies were streaked onto homologous media for isolation and subsequently grown on the PTYG medium.

Four bacterial types obtained from American Type Culture Collection were included with the above isolates in further experimentation. These included *Micrococcus luteus* (ATCC 4698), *Pseudomonas putida* (ATCC 17484), *Rhodococcus rhodochrous* (ATCC 14347) and *Arthobacter globiformis* (ATCC 8010). Another bacterial isolate, termed NP-Alk, was also included in these studies. This gram negative bacterium was isolated from petroleum-contaminated soil, is able to utilize naphthalene as the sole carbon and energy source, is peritrichously flagellated and tentatively identified as an *Alcaligenes*

species (14).

Characterization of Bacterial Isolates- Each bacterial isolate was evaluated by several methods to identify the bacterial types capable of surviving treatment of soil with HDTMA.

Motility of each bacterial isolate was determined by inoculating 0.5% agar tubes of PTYG media with a loopful of cells, allowing the cells to grow and visually observing the growth pattern in the soft agar. Motile bacteria move away from the initial site of inoculation, forming a hazy cloud of growth. Nonmotile bacteria form a dense layer of cells at the site of inoculation.

The presence of endospores was examined for each isolate by heating a cell suspension in a water bath at 80C for ten minutes with subsequent plating onto PTYG agar plates. Growth indicates that the cell line is capable of producing endospores. Phase-contrast microscopy of stationary phase broth cultures also indicated isolates able to produce spores.

Colony morphology was evaluated for each isolate after growth on PTYG agar plates. Color, size and morphological shape were noted. Cellular shapes were evaluated under a phase contrast microscope as wet mounts prepared from PTYG agar plates. Cells were evaluated with respect to cellular shape and other distinguishing characteristics.

The presence of the catalase enzyme was determined for each isolate. A drop of 3% H_2O_2 was placed onto a glass slide followed by a loopful of cells from a single colony, followed by a glass coverslip. A catalase positive bacterium was indicated by the presence of bubbling

and a catalase negative bacterium identified by the absence of bubbling. The oxidase enzyme was determined for each isolate as well. A drop of 1% N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Sigma) was placed onto filter paper followed by a loopful of cells from a single colony. An oxidase positive bacterium induced a purple coloration and an oxidase negative bacterium caused no color change.

Biolog and FAME analyses were conducted (Biolog Inc., Hayward, Ca.) on each bacterial isolate to obtain a best fit to a known genus or species. Biolog is an analysis of 95 organic substrates that are able to be utilized by bacteria, compares the substrate utilization pattern to a library of data for known bacterial types and uses this information to determine the genus and species of the unknown bacterium. FAME represents a fatty acid methyl ester analysis and compares the resultant fatty acid profile of the unknown bacterium with that of a known bacteria to give best matches.

Hydrophobicity Determination- The hydrophobicity of each bacterial isolate was measured by hydrophobic interaction chromatography using CL-4B gel (Pharmacia). Cultures were grown in PTYG liquid media containing 5000 dpm/ml [UL-^{14}C]-glucose (8.7 mCi/mmol) to early stationary phase before harvesting of ^{14}C-labeled cells. Each culture was centrifuged (6,000 x g, 5 minutes), the pellet washed several times with a PBS solution and recentrifuged. The resultant pellet was resuspended in PBS to give a final cell concentration of approximately 10^9 cells per ml. 1 ml of this cell suspension was added to 10 ml of scintillation fluid and analyzed for radioactivity by LSC. Columns were prepared in 5.75 inch

pasteur pipettes plugged with glass wool. A 1:2 dilution of CL-4B packing material with PBS was introduced into the column to a length of 3 inches. Columns were washed with 5 ml PBS before application of the radiolabeled cell suspension. To the top of the washed column, 1 ml of the cell suspension was applied and the column eluted with five 2 ml aliquots of PBS. Each 2 ml aliquot was collected in scintillation vials which contained 10 ml scintillation fluid. After a 24 hour equilibration in the dark to allow chemiluminescence to subside, samples were analyzed for radioactivity as described above. The fraction of cells eluted and retained on the gel were calculated by difference.

Hydrophobicity values were calculated using the following equation:

$$H = \log[(\text{fraction retained on gel})/(\text{fraction eluted from gel})].$$

More negative values indicate more hydrophilic cell strains and more positive values more hydrophobic cell strains.

Toxicity Assays- The toxicity of several quaternary ammonium cations to bacterial isolates was determined by incubating a suspension of cells in PBS containing a known concentration of cation followed by dilution and plating. The organic cations tested included HDTMA, nonyltrimethyl-ammonium (NTMA), dodecyltrimethylammonium (DdTMA), cetylpyridinium (CPB) and dimethyldioctadecylammonium (DODMA). Structures for these cations are depicted in Figure 2.1..

All bacterial cultures were grown in PTYG media, or nutrient media

for *Pseudomonas putida* (17484) only, formulated as described above. Cells were harvested at early stationary phase, centrifuged (6,000 x g, 5 minutes) and washed several times in PBS. Cell pellets were resuspended in PBS to give a concentration of $2x10^9$ cells per ml. Organic cation solutions were prepared in PBS at twice the desired testing concentration. For instance, to test the toxicity of 50 uM HDTMA a 100 uM HDTMA solution was prepared in PBS. To sterile dilution tubes, 1 ml of the cell suspension and 1 ml of the organic cation solution were added to give a final cell concentration of $1x10^9$ cells per ml and the desired concentration of organic cation. This suspension was incubated at room temperature for exactly one hour before dilution in PBS and plating onto PTYG or nutrient agar plates for counting. Results are calculated as percent survival with the no treatment controls representing 100%.

The toxicity of the five quaternary ammonium cations listed above were measured for *Pseudomonas putida* (17484) to determine differences of toxicity between the cations. Similarly, toxicity assays were conducted for HDTMA utilizing the 11 bacterial strains isolated from an HDTMA-treated soil, four strains obtained from ATCC and NP-Alk to determine HDTMA toxicity to various types of organisms. The concentration at which 50% lethality was observed (LC_{50}) was calculated for each bacterial strain by performing linear regression of the log [HDTMA] at which an effect was observed and the percent survival of the bacterium tested.

An experiment was conducted to determine the effect of adding smectite clay to HDTMA solutions on the survival of *Pseudomonas putida*

(ATCC 17484). Increasing amounts of smectite clay was added to a 200 uM HDTMA solution in PBS to remove HDTMA from solution. To the clay-HDTMA suspensions, 1 ml of the *Pseudomonas putida* (ATCC 17484) cell suspension was added at a concentration of $2x10^8$ cells per ml. Tubes were incubated at room temperature for exactly one hour, diluted in PBS and plated onto PTYG agar media for counting.

A time course for HDTMA toxicity was conducted using *Pseudomonas putida* (ATCC 17484) to determine if HDTMA toxicity is instaneous or exerted over a period of time. A 20 uM HDTMA solution was prepared in PBS and 1 ml aliquots were added to 1 ml aliquots of the cell suspension at a concentration of $2x10^8$ cells per ml. The final concentration of HDTMA was 10 uM; each tube contained 1 x 10^8 cells per ml. Tubes were incubated at room temperature for time intervals of 15, 30, 45 and 60 minutes before dilution in PBS and plating onto PTYG agar media for counting.

HDTMA Sorption Isotherms to Bacterial Isolates- HDTMA uptake by each bacterial strain was measured to correlate the magnitude of toxicity with the uptake of HDTMA from solution by the cells. HDTMA isotherms were conducted using the batch equilibrium technique. Cultures were grown to early stationary phase in PTYG liquid media, harvested by centrifugation (6,000 x g, 5 minutes), washed several times with PBS and repelleted. The resultant cell pellet was resuspended in a small volume of PBS and 1 ml added to glass Corex tubes that were equipped with teflon-lined screw caps. The cell suspensions were centrifuged again (6,000 x g, 5 minutes) and the supernatants removed, leaving the cell

pellets. Solutions of HDTMA were prepared in PBS with unlabeled HDTMA and sufficient ^{14}C-HDTMA to attain initial activities of approximately 5,000 dpm/ml. 1 ml of each solution was added to 7.5 ml scintillation counting cocktail and analyzed as described previously to measure initial activities. 25 ml of each solution was added to tubes containing cell pellets and incubated on a rotary shaker (room temperature, 1 hour). The cell suspensions were centrifuged (6,000 x g, 10 minutes), 1 ml of the supernatant added to 7.5 ml scintillation counting cocktail and analyzed for activity, and the concentration of HDTMA in solution was determined. The concentration of HDTMA bound to the cells was determined by difference. The distribution coefficient, K_d, was calculated using the Freundlich equation by plotting the log concentration of sorbed HDTMA versus the log concentration of HDTMA remaining in solution; where K_d equals the inverse log of the y-intercept of this plot.

RESULTS

HDTMA adsorption isotherms were conducted using the Marlette A horizon soil (Figure 2.1) and the Eielson aquifer material (Figure 2.2). The adsorption isotherms were characterized by a steep initial rise when the amount of HDTMA added was less than the CEC of the soil followed by a plateau near the measured CEC. This is characteristic of HDTMA isotherms onto soil materials (4, 18), showing the strong affinity of the soil for HDTMA resulting in a nearly stoichiometric displacement of native inorganic exchange ions by HDTMA. The aqueous phase concentration of HDTMA could be predicted from these isotherms for a given level of HDTMA addition, which is expressed hereafter as a percent of the soil's CEC.

Treatment of the freshly collected Marlette A horizon soil and Eielson aquifer material at 50% of the CEC with HDTMA for exactly one hour prior to dilution and plating onto a variety of solid media served as our isolation procedure for heterotrophic bacteria able to survive. A no treatment control was included in the experiment for comparative purposes to indicate the reduction in the overall diversity and size of the aerobic bacterial population by treatment of the soil with HDTMA. Table 2.1 displays the bacterial numbers present and diversity of the aerobic bacterial population with and without HDTMA treatment. Generally, a one log reduction in cell densities resulted from treatment

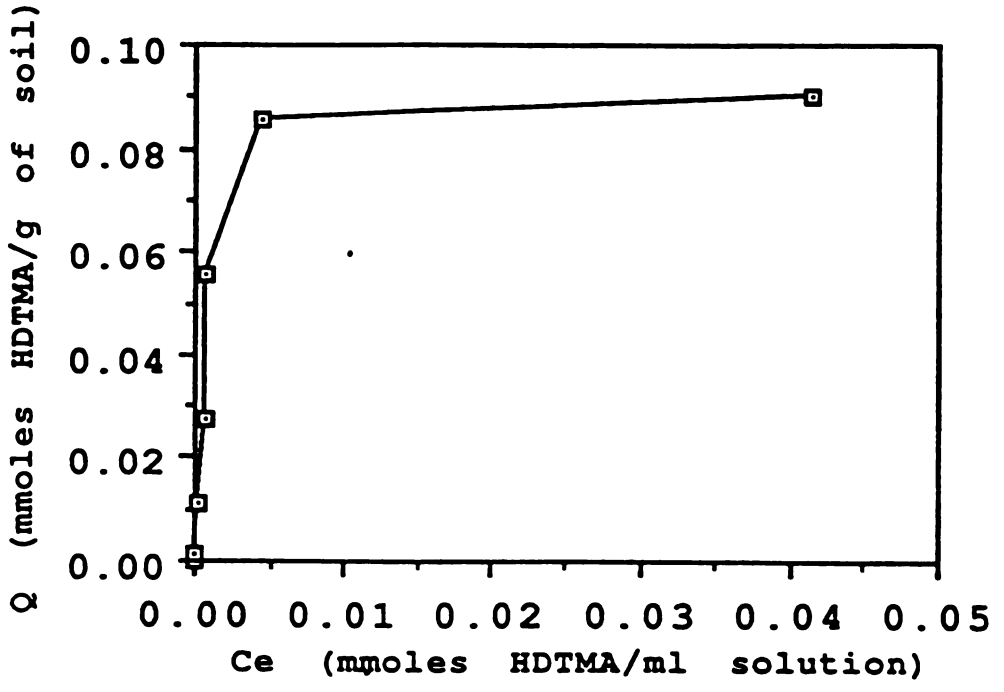

Figure 2.1: HDTMA adsorption isotherm to the Marlette A horizon soil by the batch method.

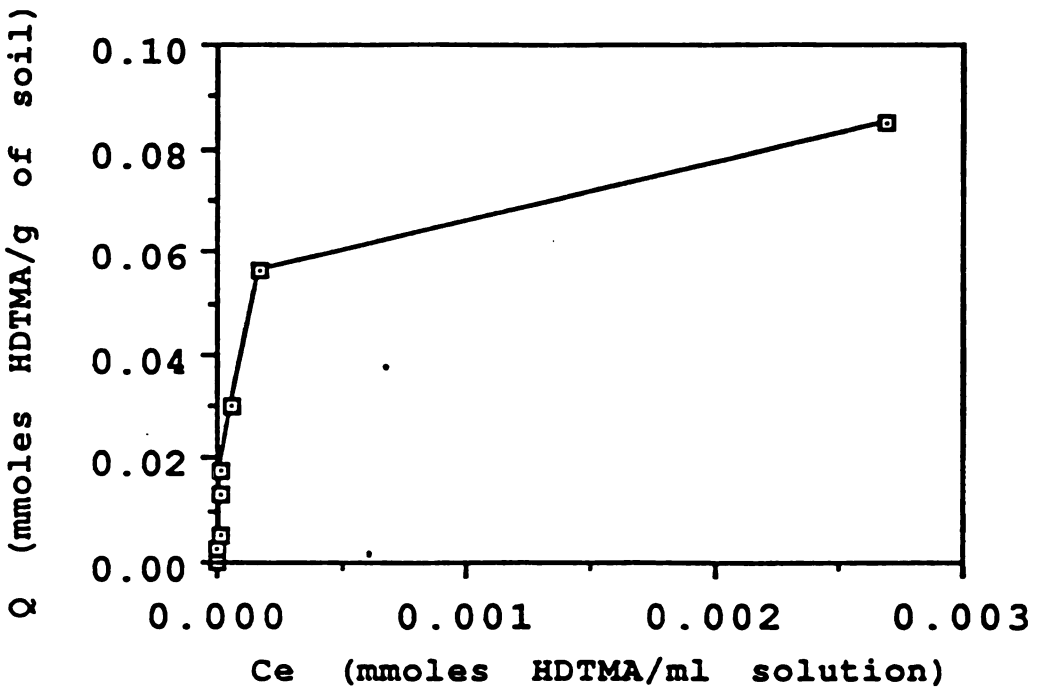

Figure 2.2: HDTMA adsorption isotherm to the Eielson aquifer material by the batch method.

Table 2.1: Microbial numbers and diversity in soils untreated and treated with HDTMA.

Soil	Media[a]	Treatment[b]	# Colony Types	# Bacteria[c]
Elelson	1	control	14	$7.73x10^5$
	1	HDTMA	4	$4.00x10^4$
	2	control	12	$2.86x10^5$
	2	HDTMA	5	$1.66x10^4$
	3	control	17	$9.73x10^5$
	3	HDTMA	7	$6.73x10^4$
	4	control	5	$5.46x10^5$
	4	HDTMA	2	$5.21x10^4$
Marlette	1	control	18	$2.09x10^6$
	1	HDTMA	9	$5.27x10^5$
	2	control	14	$9.39x10^5$
	2	HDTMA	6	$5.91x10^4$
	3	control	20	$3.14x10^6$
	3	HDTMA	9	$7.21x10^5$
	4	control	5	$6.09x10^5$
	4	HDTMA	3	$5.16x10^4$

[a]Media 1 corresponds to Nutrient, media 2 to PTYG, media 3 to 1:20 PTYG and media 4 to Tap Water.
[b]Controls were incubated in PBS, HDTMA treatments at 50% saturation of the CEC, each for one hour prior to plating.
[c]Represents the number of viable bacteria per gram of soil, dry weight.

of the soil with HDTMA. Diversity (number of bacterial types) of the aerobic bacterial population was generally reduced by at least 50% in the HDTMA-treated samples.

Bacterial colonies obtained from the above procedure for the Marlette A horizon soil were streaked for purity on homologous media. Eleven isolates were obtained and characterized with respect to standard microbiological tests such as gram staining, presence of catalase and oxidase enzymes, motility and spore formation. The results of each of these tests as well as the cellular and colony morphologies for each bacterial isolate are given in Table 2.2.

Biolog and Fame designations for each bacterial isolate are depicted in Table 2.3. Several designations are listed for isolates where replicate determinations were made with differing results or multiple designates were obtained from a single assay. Although several of the isolates return identical genus and species designations from the Biolog and FAME analyses, each isolate is different due to differences in growth characterstics on solid media and other distinguishing characteristics. Considering the characteristics of each bacterial isolate displayed in Table 2.2 with the FAME and Biolog designations shown in Table 2.3, isolates A through O and Y belong to the genus *Bacillus*. Although it is difficult to apply a unique species designation to every *Bacillus* isolate, each can be considered unique due to distinguishing growth and morphological characteristics. Exclusion of these isolates from other genus designations given in the FAME and Biolog analyses was appropriate due to the characteristics in morphology

Table 2.2: Characteristics of bacterial isolates from HDTMA-treated Marlette A horizon soil.

Isolate	Colony Morph.	Cell Morphology	Motility
A	Rough,Tan	Lg. Rods,Pairs	-
C	Rough,White	Lg. Rods,Fours	-
D	Sprawling,Tan	Lg. Rods,Chains	-
F	Round,Brown	Lg. Rods,Pairs	-
H	Rough,White	Lg. Rods,Single	-
J	Round,Br.Red	Sm. Rods,Single	+
O	Rough,White	Lg. Rods,Chains	+
P	Opaque,Slimy	Sm. Rods	+
W	Round,Orange	Lg. Rods,Single	+
X	Round,Yellow	Sm. Cocci	-
Y	Round,Yellow	Rods,Single	+

Isolate	Gram St.	Spore Form.	Oxidase	Catalase
A	+	+	-	+
C	+	+	-	+
D	+	+	-	+
F	+	+	-	+
H	+	+	-	+
J	-	-	-	+
O	+	+	+/-	+
P	+	-	-	+
W	+	-	-	+
X	+	-	-	+
Y	+	+	-	+

Table 2.3: FAME and Biolog analysis results for bacterial isolates from HDTMA-treated Marlette A horizon soil.

Isolate	FAME designations[a]	Biolog designations[b]
A	*B.megaterium* (0.688)	*Coryn.jeikeium* (0.539)
	B.laterosporus (0.26)	*B.insolitus* (0.096)
C	*B.metaterium*(0.716)	*B.insolitus* (0.50)
	Kurthia gibsonii (0.015)	*Coryn.jeikeium* (0.404)
D	*B.megaterium* (0.541)	*B.insolitus* (0.548)
	B.subtilus (0.19)	
	B.amyloliquiefaciens(0.19)	
	B.aminovorans (0.171)	
F	*B.megaterium* (0.733)	*B.insolitus* (0.580)
H	*B.mycoides* (0.156)	NO MATCH
O	*B.gordonae* (0.592)	Coryn.pseudodiph.(0.509)
		B.insolitus (0.408)
P	*Entero.cancero.*(0.42)	*Entero.asburiae* (0.64)
	Kluyvera cryocresc.(0.25)	
W	NO MATCH	NO MATCH
X	*Micrococcus luteus* (0.71)	*B.insolitus* (0.814)
	Arthro.protophormiae(0.682)	
	B.coagulans (0.297)	
	Arthro.uratoxydans (0.287)	
Y	*B.filicolonicus* (0.184)	*B.megaterium* (0.577)
	B.pantothenticus (0.015)	
	B.licheniformis (0.021)	

[a]Fatty Acid Methyl Ester analysis of bacterial isolates with values indicating similarity coefficients to given bacteria.
[b]Biolog analysis of bacterial isolates with values indicating similary coefficients to given bacteria.

and growth. Isolate X may be considered a *Micrococcus luteus* due to the very high similarity coefficient obtained from the FAME analysis and the observation of small coccoid-shaped cells, as well as the lack of spore formation eliminating it from the *Bacillus* designation obtained in the Biolog analysis. Isolate P likely belongs to the genus *Enterobacter* resulting from the high correlation coefficients in the FAME and Biolog analyses. Isolate W did not return a match with either analysis, indicating a very unique bacterium. Isolate J was unable to be analyzed due to difficulties culturing the bacterium in a media compatible with the analysis.

The LC_{50}, which corresponds to the concentration of HDTMA at which 50% of the bacteria are able to survive over a one hour exposure period, for each bacterial isolate indicate a wide response range to HDTMA (Table 2.4). Low uM concentrations of HDTMA affected the survival of many of the bacterial isolates. The LC_{50} values generally ranged from 7 uM to 100 uM, with one isolate having an LC_{50} greater than 500 uM HDTMA.

Pseudomonas putida (ATCC 17484), the bacterium with the lowest LC_{50} for HDTMA, was used in further experimentation to compare the relative toxicities of other alkyl quaternary ammonium cations. Figure 2.3 illustrates the percent survival of *Psuedomonas putida* in various concentrations of a C-9 (NTMA), C-12 (DdTMA), and C-16 (HDTMA) monoalkyl trimethylammonium cations, a C-16 (CPB) monoalkyl pyridinium cation, and a C-18 (DODMA) dialkyl dimethylammonium cation. For the monoalkyl compounds, toxicity increased in the order NTMA<DdTMA<HDTMA. Toxicities

Table 2.4: Physiological properties of bacterial isolates.

Bacterium	Hydrophobicity	K_d[a]	HDTMA LC_{50}[b]
A	0.717	9473	44.0
C	0.166	18985	46.0
D	2.571	6306	514.0
F	-0.376	10785	61.0
H	-0.099	2575	54.0
J	-0.449	1662	7.2
O	-1.266	6275	46.0
P	-1.074	2293	51.0
W	1.045	11857	51.0
X	-1.142	656	28.0
Y	0.539	9913	28.0
P.putida	0.155	14298	4.0
M.luteus	2.060	1461	53.0
R.rhodochrous	0.355	7167	37.0
A.globiformis	-0.768	439	7.0
NP-Alk	-0.280.	17523	8.5

[a]Value represents the distribution coefficient of HDTMA between the aqueous phase and the bacterium.
[b]Value represents the concentration of HDTMA in uM at which 50% of the bacteria are killed.

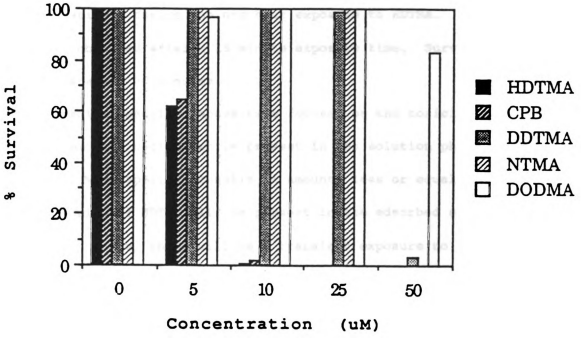

Figure 2.3: Toxicity of quaternary ammonium cations with varying alkyl chain lengths to *Pseudomonas putida* (ATCC 17484).

of the two C-16 alkyl compounds, HDTMA and CPB, were similar. The dialkyl compound, DODMA, was the least toxic of those tested.

The toxicities described above were conducted for one hour exposure times. A time course study was conducted utilizing Pseudomonas *putida* (17484) to determine the temporal toxicity of HDTMA. Figure 2.4 illustrates the % survival of the bacterium in a 10 uM HDTMA solution at given time periods up to one hour. It was found that the toxicity occurs progessively during the one hour exposure to HDTMA. A 60% survival was observed after a 15 minute exposure time. Survival was less than 10% after 30 minutes.

The results described above have focused on the toxicity of quaternary ammonium cations while present in the solution phase as the free cation. When applied to soils in amounts less or equal to the CEC, the vast majority of HDTMA will be present in the adsorbed state (Figure 2.1 and 2.2). Hence, there will be a transient exposure to free aqueous phase HDTMA, followed by a long term exposure to soil-bound HDTMA where toxicity may be substantially different. To address this issue, a 100 uM HDTMA solution was prepared and various amounts of smectite clay were added resulting in different amounts of free and bound HDTMA. The toxicity of the clay-water-HDTMA mixutures to *Pseudomonas putida* (17484), the bacteria towards which HDTMA was the most toxic in the free state, was evaluated. Figure 2.5 displays the percent survival of the bacterium versus the amount of smectite clay added to the system, and the calculated aqueous phase HDTMA concentration assuming the stoichiometric exchange of HDTMA on the clay. The results show HDTMA toxicity to the bacterial strain tested can be completely eliminated by

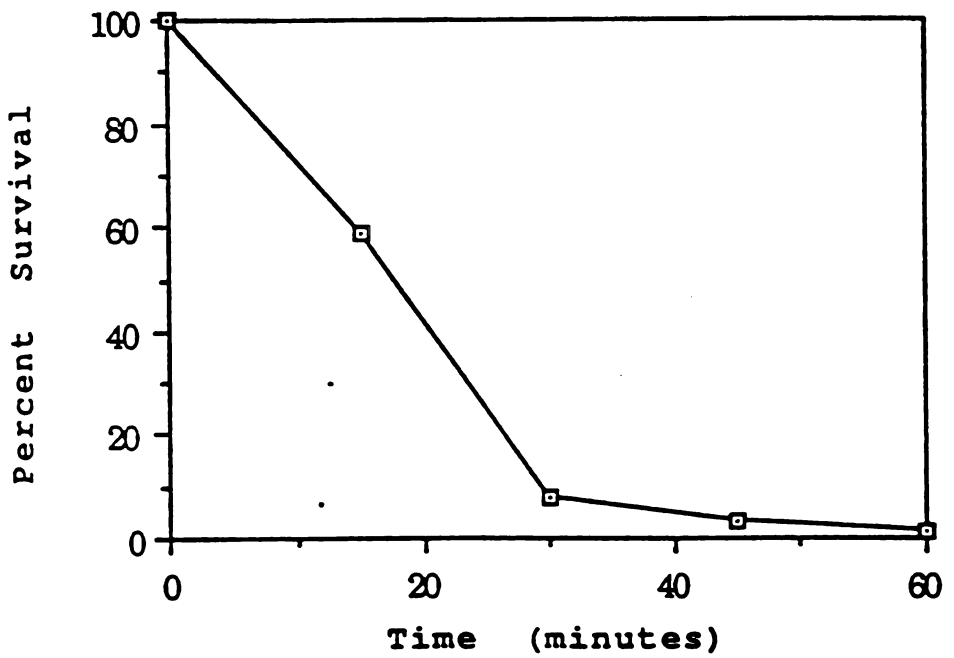

Figure 2.4: Time course effect of HDTMA on survival of *Pseudomonas putida* (ATCC 17484).

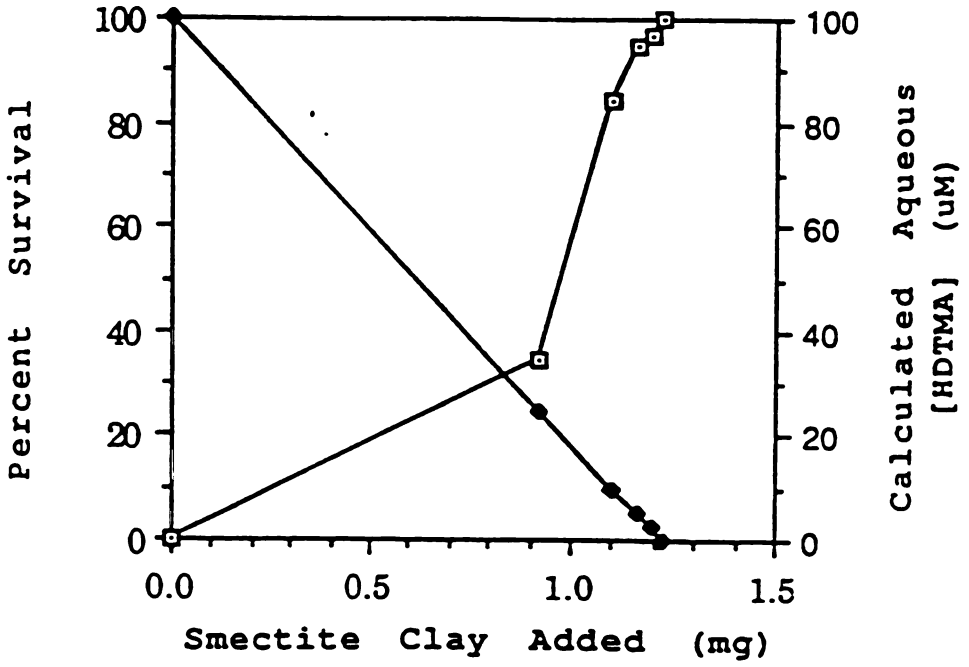

Figure 2.5: Alleviation of HDTMA toxicity to *Pseudomonas putida* (ATCC 17484) by addition of smectite clay.

adding smectite clay. When the calculated concentration of aqueous HDTMA was reduced from 100 uM to 25 uM, the survival increased from 0 to 25 percent. Interestingly, at 25 uM HDTMA in the absence of smectite clay, a 0% survival was observed (Figure 2.3). Similarly, at a calculated aqueous concentration of 10 uM in the clay-water mixture, a 90% survival of *Pseudomonas putida* was observed, whereas a 0% survival was noted at 10 uM HDTMA without smectite.

The hydrophobicity of each bacterial strain was determined by hydrophobic interaction chromatography. Table 2.4 presents the values from this study, a negative value indicating a hydrophilic cell and a positive value indicating a hydrophobic cell strain. The bacterial strains tested represent a wide range of hydrophobicity/philicity values. We attempted to relate this characteristic to HDTMA resistance in the different cell lines, as expressed by the LC_{50} values. Linear regression gave an R^2 value of 0.05, indicating no relationship between these parameters.

The distribution coefficient, K_d, of HDTMA for several of the bacterial isolates was measured. The degree of uptake of HDTMA from the aqueous solution by each isolate could be assigned a value for comparison between several bacterial types. Table 2.4 depicts the measured K_d values, where a high K_d indicates a high degree of sorption of the HDTMA from solution. The measured K_d values ranged for approximately 400 to 19,000 indicating a substantial (nearly 50-fold) difference in the affinity of the different bacterial isolates for HDTMA. Correllation of the measured K_d value with the LC_{50} of HDTMA for

a given isolate was attempted and an R^2 value of 0.08 was obtained indicating no relationship between these parameters.

DISCUSSION

Modification of the Marlette A horizon soil at a level of 50% saturation of the cation exchange capacity with HDTMA resulted in a one log reduction in total numbers and a reduction from 12 to 16 bacterial types in the no-treatment control to only a few types present in the HDTMA treated soil (Table 2.1). The observed reduction in the size and diversity of the population is evidence of the general toxicity of quaternary ammonium cations such as HDTMA to bacterial species.

Isolation and characterization of the bacteria able to survive modification of the soil with HDTMA showed that seven out of 11 of the isolates belonged to the genus *Bacillus*, gram positive rods that are able to form resistant spores (Table 2.2 and 2.3). Other gram positive isolates and a gram negative isolate were also observed and genus designations depicted in Table 2.3. Distinguishing characterstics of each isolate (i.e. catalase, oxidase, motility, spore formation) further reinforce assigning each isolate to a genus. Research has shown that quaternary ammonium compounds have a selective pressure favoring gram positive cell strains (12). The ability of *Bacilli* to form resistant spores apparently enables them to further resist the toxicity of HDTMA.

Measurement of the LC_{50} of *Pseudomonas putida* (ATCC 17484) for aqueous phase HDTMA indicated that this bacterium was very susceptible to HDTMA with an LC_{50} of approximately 5 uM. Further studies including various monoalkyl quaternary ammonium cations indicated the toxicity

decreased as the size of the alkyl moiety decreases from C-16 to C-12 to C-9. The dialkyl (C-18) QAC, DODMA, was less toxic than any of the monoalkyl quaternary ammonium cations tested. Toxicity of the C-16 trimethylammonium cation (HDTMA) and the C-16 pyridinium cation (CPB) were similar suggesting that toxicity was related to the alkyl moiety and not the polar head group. Previous studies have indicated that the increased toxicity of long chain alkyl ammonium cations may be a result of the increased hydrophobicity of the molecules and their ability to form dimers (12), which were presumed to be more suitable to traverse the cellular membranes. DODMA, a dialkyl (C-18) quaternary ammonium cation, was found to be the least toxic to *Pseudomonas putida* (ATCC 17484), perhaps a result of the inability of this molecule to interact with the bacterial membrane due to conformational constraints resulting from intramolecular interactions of the C-18 moieties, or its lower critical micelle concentration. The relatively nontoxic characteristic of DODMA to bacteria as compared to HDTMA suggests its utility for coupling enhanced contaminant immobilization in quaternary ammonium cation-treated soils with biodegradation of the immobilized organic contaminants.

Information on the bacterial types that survived in soil treated with HDTMA was obtained. This characterization included measurement of the LC_{50} for HDTMA, the distribution coefficient (K_d) between the solution phase and sorption to bacterial cells, and the cellular hydrophobicity for fifteen bacterial isolates including 11 that were isolated from HDTMA-treated soil. A wide range of LC_{50} values for bacteria exposed to HDTMA were observed indicating that some bacteria

were better able to survive in HDTMA solutions. However, since this parameter ranged by well over an order of magnitude (7 to 514 uM) it does not seem to be a singular indicator of survivability in HDTMA-treated soils.

To evaluate the possibility of the bacterial hydrophobicity being involved in susceptibility to HDTMA, measurement of the cellular hydrophobicity of the fifteen bacterial isolates was performed. A wide range of values were observed, but attempts to link HDTMA toxicity with cellular hydrophobicity gave little correlation. An R^2 value of 0.05 was obtained, indicating no obvious relation between the cellular hydrophobicity and HDTMA toxicity.

Uptake of HDTMA from solution was measured by conducting batch isotherms with each bacterial isolate. The distribution coefficient, K_d, was computed from the resultant isotherm for each bacterial isolate. Linear regression of the LC_{50} values for each isolate versus their K_d for HDTMA indicated little correllation between the two parameters (R^2 value of 0.08) as was found between the LC_{50} and cellular hydrophobicity.

Toxicity of aqueous phase HDTMA to the bacterial isolates tested gives information on the initial toxic effects to soil bacteria associated with HDTMA additions. However, these effects are likely to be transient because most HDTMA added to soil will become bound to soil components, chiefly cation exchange sites of clay minerals, thus altering the bioavailability and hence toxicity of HDTMA. Additions of smectite clay to HDTMA suspensions with *Pseudomonas putida* indicated that relatively low amounts of smectite clay added to the solution alleviated the toxicity of HDTMA. This reduction in HDTMA toxicity

predominantly occurs due to the lowered aqueous concentration after adsorption of the HDTMA molecules from the solution onto the clay mineral through ion-exchange interactions. There also appears to be some secondary benefit of smectite for reducing HDTMA toxicity. Even assuming that HDTMA adsorbed stiochiometrically to the exchange sites of smectite, the calculated aqueous phase HDTMA was less toxic than the same HDTMA concentration in a clay free system. Although the cause of this secondary effect is unknown, it is clear that once bound to clay, the toxicity of HDTMA is essentially eliminated.

Measurement of the rate at which HDTMA exerts toxicity on *Pseudomonas putida* (ATCC 17484) revealed that most of the toxicity occurs within the first 30 minutes of exposure. However, only about 40% of the toxicity was expressed during the first 15 minutes of exposure. Ion exchange reactions of HDTMA with subsoils has been shown to be rapid (>95% in 12 min.) with very high selectivity coefficients (up to 10^7 to 10^8) (32). The rapid binding of HDTMA, the substantially reduced toxicity of bound HDTMA, and the temporal dependance of HDTMA toxicity suggest that toxicity of aqueous phase HDTMA to bacteria will not be fully expressed when HDTMA is added to soil.

CONCLUSIONS

This study indicates that modification of soil with HDTMA, a quaternary ammonium cation, to reduce mobility of organic contaminants initially has a detrimental effect on the numbers and diversity of the heterotrophic bacterial population. Bacterial isolates obtained from an HDTMA-treated soil each have different response to aqueous phase HDTMA, indicating that there are other differences among the bacteria tested affording resistance to HDTMA. Attempts to correllate cellular hydrophobicity and the HDTMA cell-water distribution coefficient with the LC_{50} for HDTMA and each bacterium showed little correllation between these parameters. The most prevalent viable bacteria present in HDTMA-treated soils were gram positive spore-formers belonging to the genus *Bacillus*.

Although HDTMA was found to be highly toxic to bacteria while present in the free cationic state, HDTMA applied to soil systems will predominantly be present in the bound form through ion exchange interactions with chiefly clay minerals. HDTMA bound to smectite clay was relatively nontoxic. Additionally, the toxic effects of HDTMA are not fully expressed until between 30 and 60 minutes of exposure, thus allowing time of HDTMA added to soil to become adsorbed and hence rendered less toxic.

The effects of HDTMA additions to soils and subsoils is likely to occur in two phases. First, during the time required for HDTMA to

displace exchangeable cations in soil and subsoils and become bound, an initial detrimental effect of HDTMA on the bacterial population will occur. Once bound, repopulation of an HDTMA-modified zone by surviving bacteria within the treated zone and by bacteria from surrounding untreated areas through movement with groundwater flow or other means will probably occur as the soil-bound HDTMA is relatively nontoxic. DODMA, a large dialkyl quatérnary ammonium cation, was found to be significantly less toxic than HDTMA and represents a possible alternative to HDTMA. These results suggest that soil modification with quaternary ammonium cations to reduce contaminant mobility, coupled with biodegradation of sorbed contaminants, is a feasible and potentially useful remediation approach.

REFERENCES

1. Barzu, Octavian, Guerrieri, Ferruccio , Scarfo, Rosanna, Capossa, Giuseppe and Papa, Sergio. 1989. Effect of cetyltrimethylammonium on ATP hydrolysis and proton translocation in the F_0-F_1 H^+ ATP synthase of mitochondria. J. of Bioenergetics and Biomembranes 21:403-414.

2. Beaubien, A., L. Keita, and C. Jolicoeur 1987. Flow microcalorimetry investigations of the influence of surfactants on a hetergeneous aerobic culture. App. Environ. Micro. 53:2567-2573.

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

4. Boyd, S.A., M.M. Mortland, and C.T. Chiou. 1988. Sorption characterstics of organic compounds on hexadecyltrimethyl-ammonium-smectite. Soil Sci. Soc. Am. J. 52:652.

5. Boyd, S.A., J.F. Lee, and M.M. Mortland. 1988. Attenuating organic contaminant mobility by soil modification. Nature. 333:3451.

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

7. Cabral, Joao P.S. 1991. Mode of antibacterial action of dodine (dodecylguanidine monoacetate) in *Pseudomonas syringae*. Can. J. Microbiol. 38:115-123.

8. Chiou, C.T., L.J. Peters, and V.H. Freed. 1981. Soil-water equilibria for nonionic organic compounds. Science. 213:684.

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

10. Coats, G.E., H.H. Funderburk Jr., J.M. Lawrence, D.E. Davis. 1966. Factors affecting persistence and inactivation of diquat and paraquat. Weed Res. 6:58-66.

11. Federle, Thomas W. and Roy M. Ventullo. 1990. Mineralization of surfactants by the microbiota of submerged plant detritus. App. Environ. Micro. 56:333-339.

12. Gilbert, P. and A. Al-Taae. 1985. Antimicrobial activity of some alkyltrimethylammonium bromides. Letters in App. Micribiol. 1:101-104.

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

14. Guerin, W.F. and S.A. Boyd. 1992. Differential bioavailability

of soil-sorbed naphthalene to two bacterial species. AEM. 58:1142-1152.

15. Jenkinson, D.S. 1966. Studies on the decomposition of plant material in soil. II. Partial sterilization of soil and the soil biomass. J. Soil. Sci. 17:280-302.

16. Karickhoff, S.W. 1984. Organic pollutant sorption on aquatic systems. J.Hydraul.Eng. 110:707-735.

17. Larson, R.J. and R.D. Vashon. 1983. Adsorption and biodegradation of cation surfactants in laboratory and environmental systems. Devl. Indus. Micro. 24:425-434.

18. 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. 34:1365.

19. Lee, J.F., M.M. Mortland, C.T. Chiou, D.E. Kile and S.A. Boyd. Adsorption of benzene, toluene, and xylene by two tetramethylammonium-smectites having different charge densities. Clays and Clay Min. 38:113-120.

20. Marchesi, Julian R., Nicholas J. Russell, Graham F. White and William A. House. 1991. Effects of surfactant adsorption and biodegradability on the distribution of bacteria between sediments and water in a freshwater microcosm. App. Environ. Micro. 57:2507-2513.

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

22. Rhoades, J.D. 1982. Cation Exchange Capacity, p. 149-157. *In* A.L. Page, R.H. Miller, and D.R. Keeney(ed.), Method of soil analysis, part 2. American Society of Agronomy, Madison, Wis.

23. Shimp, Robert J., Schwab, Burney S., and Robert J. Larson. 1989. Adaptation to a quaternary ammonium surfactant by suspended microbial communities in a model stream. Environ. Tox. Chem. 8:723-730.

24. Shimp, Robert J., and Young, Richard L. 1987. Availability of organic chemicals for biodegradation in settled bottom sediments. Ecotox. and Environ. Safety. 15:31-45.

25. Tennant, Jan M., Bruce R. Lyon, Melvin Midgley, Gwyn Jones, Amarjit S. Purewal, and Ronat A. Skurray. 1989. Physical and Biochemical Characterization of the qacA gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. J. Gen. Micro. 135:1-10.

26. Theng, B.K.G. The chemistry of clay-organic reactions, pp. 221-238 (Wiley, New York, 1974).

27. Tubbing, Diny M.J., and Wim Admiraal. 1991. Inhibition of bacteria and phytoplanktonic metabolic activity in the lower River Rhine by ditallowdimethylammonium chloride. Appl. Environ. Micro. 57:3616-3622.

28. Van Leeuwen, K., C. Roghair, J. de Greef, and T.de Nijs. 1990. Wasverzachters. II. Resultaten van aanvullend onderzoek. Water 11:295-299.

29. 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.

30. Wszolek, Patricia C., and M. Alexander. 1979. Effect of desorption rate on the biodegradation of n-alkylamines bound to clay. J. Agric. Food Chem. 27:410-414.

31. Wu, S., and P.M. Gschwend. 1986. Sorption kinetics of hydrophobic organic compounds to natural sediments and soils. Environ. Sci. Technol. 20:717-725.

32. Xu, S. and S.A. Boyd. 1992. Soil modification for enhanced removal of organic contaminants from aqueous solutions: Hexadecyltrimethylammonium (HDTMA)-soil interactions. Agronomy Abstracts.

APPENDIX

APPENDIX A

Structure of Quaternary Ammonium Cations

HDTMA $CH_3(CH_2)_{15}N^+(CH_3)_3$

DdTMA $CH_3(CH_2)_{11}N^+(CH_3)_3$

NTMA $CH_3(CH_2)_8N^+(CH_3)_3$

DODMA $[CH_3(CH_2)_{17}]_2N^+(CH_3)_2$

CPB