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(Eds.)

Soil Mineral-Microbe-Organic Interactions

Theories and Applications

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Qiaoyun Huang · Pan Ming Huang ·
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ISBN: 978-3-540-77685-7 e-ISBN: 978-3-540-77686-4

Library of Congress Control Number: 2008926228

Cover design: deblik, Berlin

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Preface

Minerals, organic matter and microorganisms are the major solid components in soil systems. These three constituents do not function independently but rather interact with each other constantly at all times and everywhere in the soil ecosystem. The interactions profoundly affect a series of physical, chemical and biological processes of soils including the behavior, transformation and fate of various nutrients and pollutants. The research on these interactions should, thus, be an important issue for Soil and Environmental Scientists. Therefore, the International Society of Soil Science established the Working Group MO in 1990, which was promoted to a new Commission 2.5 Soil Physical/Chemical/Biological Interfacial Interactions of the International Union of Soil Sciences (IUSS) in 2004. To date, the Working Group has sponsored four international symposia and these conferences were held in Edmonton (Canada, 1992), Nancy (France, 1996), Naples (Italy, 2000) and Wuhan (China, 2004), respectively.

The 4th International Symposium on Interactions of Soil Minerals with Organic Components and Microorganisms (ISMOM2004) was the first Inter-Congress Symposium of IUSS Commission 2.5. The conference was cosponsored by the International Union of Pure and Applied Chemistry (IUPAC). Doctors P.M. Huang (Canada), A. Violante (Italy), J. -M. Bollag (USA), J. Berthelin (France), J. Zhou (China) and Q. Huang (China) served in the Symposium Organizing Committee. The meeting attracted 135 delegates from 22 countries and regions in the world including Canada, Chile, China, France, Germany, Hong Kong SAR, India, Indonesia, Iran, Italy, Japan, Kenya, Korea, Malaysia, New Zealand, Russia, South Africa, Thailand, The Netherlands, USA, Venezuela and Zimbabwe. The theme of ISMOM2004 was "Environmental Significance of Mineral-Organic Component-Microorganism Interactions in Terrestrial Systems". The conference program was divided into the following six sessions: (1) Transformation and Dynamics of Pollutants in Soil Environments, (2) Chemical, Biological and Biochemical Processes in the Rhizosphere, (3) Bioavailability of Metals, Nonmetals and Xenobiotics Immobilized on Soil Components, (4) Distribution and Activity of Biomolecules in Terrestrial Systems, (5) Interactions between Soil Microbial Biomass and Organic Matter/Nutrient Transformations, and (6) Impact of Interactions among Soil Mineral Colloids, Organic Matter and Biota on Risk Assessment and Restoration of Terrestrial Ecosystems. There were 2 plenary lectures, 9 invited speakers, 36 oral presentations and 45 posters. Dr. N. Senesi from University of Bari, Italy, presented an IUPAC lecture entitled Metal-Humic

Substance Complexes in Soil. Dr. P. M. Huang from University of Saskatchewan, Canada, who was the founder of Working Group MO and the founding Chair of Commission 2.5 of IUSS, gave a plenary lecture on Physical-Chemical-Biological Interfacial Interactions in Soil Environments.

The 13 chapters in this book are mainly the papers from the plenary and invited speakers of ISMOM2004. They address the state-of-the-art on the theories and applications of the interactions of minerals with organic components and microorganisms in soil environments. The book presents a variety of issues on the fundamental interactions among soil minerals, organic components and microorganisms and their impacts on soil ecosystems. Part I (Chaps. 1–7) addresses the fundamentals of physical-chemical-biological interfacial reactions, the binding and transformation mechanisms of metals, metalloids, biomolecules and organic pollutants as affected by soil organic, inorganic and microbial components. Part II (Chaps. 8–13) deals with the impacts of the interactions of soil components on the dynamics of soil carbon and biomass, the bioavailability of chemicals, and on soil and environmental quality. These chapters present a variety of topics that address issues of the cutting edges of science of the subject matter. We believe that the publication of this ISMOM2004 special book would promote in-depth studies in this field for years to come. The book should be useful for research scientists, professors, graduate students, and consultants working in soil, microbial ecology and environmental sciences.

We wish to extend our gratitude to the many sponsors including the National Natural Science Foundation of China (NSFC) and Organization for the Prohibition of Chemical Weapons (OPCW). We also acknowledge the contributions from many of the Chinese Institutions such as Huazhong Agricultural University, Institute of Soil Science of the Chinese Academy of Sciences, State Key Laboratory of Agricultural Microbiology, and the Key Laboratory of Subtropical Agricultural Resources and Environment.

In addition to this book, volunteered papers presented at ISMOM2004 and accepted after rigorous external review was published as a special issue by the international journal Biology and Fertility of Soils. This special issue serves a companion volume of this IUSS- and IUPAC-sponsored book published by Springer-Verlag.

Editors: Q. Huang
P. M. Huang
A. Violante

About the Editors

Qiaoyun Huang is a professor of Soil Biochemistry at Huazhong Agricultural University, China. He received his Ph.D. degree in soil science at Huazhong Agricultural University. As a visiting scientist, he spent one year (1993–1994) in Department of Soil Science, University of Manitoba, Canada and four months (1997–1998) at University of Naples, Italy. He conducted a two-year (1998–2000) postdoctoral work in Faculty of Agriculture, Yamaguchi University, Japan.

Dr. Huang is currently Director of the Key Laboratory of Subtropical Agriculture and Environment of Ministry of Agriculture, China and Vice Chairman of the Young Scientist Association of Hubei Province. His major research is in the area of soil mineral interactions with biomolecules and microbial transformation of heavy metal in soils. He has published over 90 research articles and book chapters including 35 SCI papers and has been invited as referee for 15 international journals. He serves as a member of the editorial board of the international journal *Applied Soil Ecology* and *The Open Enzyme Inhibition Journal*. He earned an honor of Provincial Distinguished Young Scientist in 2003 and was awarded a second prize by the Ministry of Education of China for the Advancement of Natural Science in 2005.

Dr. Huang is an active participant for international conferences. He gave oral presentations at the 31th Annual Meeting of Clay Mineral Society (Saskatoon, Canada), the Second International Symposium on Interactions of Soil Minerals with Organic Components and Microorganisms (Nancy, France), the 15th and 16th International Symposia on Environmental Biogeochemistry (Wroclaw, Poland and Aomori, Japan), the 17th and 18th World Congress of Soil Science (Bangkok, Thailand and Philadelphia, USA), and the 1st International Symposium on Enzymes in the Environment (Granada, Spain). He presented an invited lecture at the 39th Annual Meeting of Indonesian Society for Microbiology. He was the Chairman of the 4th International Symposium on Interactions of Soil Minerals with Organic Matter and Microorganisms.

P. M. Huang received his Ph. D degree in Soil Science at the University of Wisconsin, Madison, in 1966. He is Professor Emeritus of Soil Science at the University of Saskatchewan, Saskatoon, Canada. His research work has significantly advanced the frontiers of knowledge on the nature and

surface reactivity of mineral colloids and organomineral complexes of soils and sediments and their role in the dynamics, transformations, and fate of nutrients, toxic metals, and xenobiotics in terrestrial and aquatic environments. His research findings, embodied in over 300 refereed scientific publications, including research papers, book chapters, and 16 books, are fundamental to the development of sound strategies for managing land and water resources.

Dr Huang developed and taught courses in soil physical chemistry and mineralogy, soil analytical chemistry, and ecological toxicology. He has successfully trained and inspired M.Sc and Ph.D. students and postdoctoral fellows, and received visiting scientists from around the globe. He has served on numerous national and international scientific and academic committees. He has also served as a member of many editorial boards such as the *Soil Science Society of America Journal, Geoderma, Chemosphere, Water, Air and Soil Pollution, and Soil Science and Plant Nutrition*. He has served as a titular member of the Commission of Fundamental Environmental Chemistry of the International Union of Pure and Applied Chemistry and is the founding Chairman of the Working Group MO "Interactions of Soil Minerals with Organic Components and Microorganisms" of the International Union of Soil Science. He received the Distinguished Researcher Award from the University of Saskatchewan and the Soil Science Research Award from the Soil Science Society of America. He is Fellow of the Canadian Society of Soil Science, the Soil Science Society of America, the American Society of Agronomy, the American Association for the Advancement of Science, and the World Innovation Foundation.

Antonio Violante is Professor of Agricultural Chemistry at the University of Naples, Italy. He received his Ph. D. in Chemistry at the University of Naples in 1969. He was awarded postdoctoral fellowships from the University of Wisconsin, USA (1967–1977) and the University of Saskatchewan, Canada (1981–1982) and was invited Visiting Professor in the Department of Soil Science, University of Saskatchewan, Canada in 1985, 1992 and 2003.

Dr Violante was Head of the Dipartimento di Scienze Chimico-Agrarie and is Coordinator of the *Doctoral School in Agrobiology and Agrochemistry* of the University of Naples Federico II. He has served on many committees of the Italian Society of Soil Science (President of the Session Soil Chemistry), and Italian Society of Agricultural Chemistry. He is vice-president and liaisons officer of Gruppo Italiano AIPEA. He was the scientific chairman and chief organizer of International and National Congresses.

Dr Violante has contributed to promote research on the interface between soil chemistry and mineralogy and soil biology. The areas of research include the formation mechanisms of Al-hydroxides and oxyhydroxides, the surface chemistry and reactivities of short-range ordered precipitation products of Al and Fe, the influence of biomolecules on the sorption/desorption of nutrients and xenobiotics on/from variable charge minerals and soils and on the factors which influence the sorption and residual activity of enzymes on phyllosilicates, variable charge minerals, organo-mineral complexes, and soils. Dr Violante is the author or co-author of 141 research articles and book chapters. He presented papers at many scientific congresses and Symposia and gave invited lectures at universities and research institutes worldwide. Dr Violante has international research/teaching experience in Canada, the US, Europe, China and Chile. He has trained students for Masters and Ph. D. Degrees and postdoctoral fellows and received visiting scientists from worldwide. He serves on the editorial board of three international journals. He is a Fellow of the Soil Science Society of America and the American Society of Agronomy.

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Part I
Fundamentals

1 Soil Physical-Chemical-Biological Interfacial Interactions: An Overview

P.M. Huang

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1 Introduction

Soil is the central organizer of the terrestrial ecosystem and its physical, chemical, and biological processes have enormous impacts on ecosystem productivity, services, integrity, and human welfare. On April 21, 2000, the Council of the International Union of Soil Sciences (IUSS) approved the organization of the IUSS scientific structure: D1. Soil in Space and Time, D2. Soil Properties and Processes, D3. Soil Use and Management,

and D4. The Role of Soils in Sustaining Society and the Environment. There are four commissions in Division D2: C2.1 Soil Physics, C2.2 Soil Chemistry, C2.3 Soil Biology, and C2.4 Soil Mineralogy. However, physical, chemical, and biological processes are not independent processes but interactive within the soil environment. Soils can be defined as complex interactive biogeochemical reactors, reservoirs of organisms, and a major compartment of the terrestrial ecosystem under the influence of anthropogenic activities.

To improve our scientific knowledge of soil resources and its application to remediation and long-term management, it is of major importance and interest to study soil organization and function, not only through the traditional subdisciplines of soil science but also through interactive approaches. The study of soil physical, chemical, and biological interfacial interactions has to be considered at different scales, namely, from molecular level to field/landscape systems and this approach is essential to stimulating further research to uncover the dynamics and mechanisms of soil processes. Therefore, a new Commission C2.5 Soil Physical/Chemical/Biological Interfacial Reactions was established in Division D2 Soil Properties and Processes within the IUSS structure. Major research thrusts of this new commission include: (1) mineral and biological catalysis and enzyme-mineral interactions leading to humus and organo-mineral complex formation; (2) surface reactions of micro- and macro-biota and biomolecules with soil particles; (3) the effect of soil abiotic and biotic interactive processes on the structure, dynamics, and activities of microbial communities; and (4) ecological impacts of soil abiotic and biotic interactive processes. This last research thrust contains two major topics: (a) porosity formation by structure or organization development; and (b) biogeochemical transformation and transport of chemical and biological components at different temporal and spatial scales.

This paper presents an overview on soil physical-chemical-biological interfacial interactions and the impacts on the terrestrial ecosystem.

2 Role of Organic Substances in the Transformation of Metal Oxides

Metal oxides have a significant role in influencing physical, chemical, and biological properties of soils. They may exist as crystalline minerals, as short-range ordered (SRO) mineral colloids, or as surface coatings on clay minerals and organic matter. Organic compounds influence the formation, transformation, and surface properties of these metal oxides. The SRO Al

and Fe oxides are among the most reactive mineral colloids in acidic and neutral soils (Huang et al. 2002; Bigham et al. 2002).

The hydrolysis and polymerization of Al and the subsequent transformation into crystalline phases are strongly influenced by the nature and concentration of natural organics (Huang and Violante 1986; Huang 1988; Krishnamurti et al. 1999, 2004). The influence of organic acids on Al transformation has been studied extensively, with most of the focus on the particular solid phases that form as a result of the perturbation of organic acids (Huang et al. 2002). The influence of a particular organic acid is generally related to the stability constant of the complex that forms with Al (Table 1). Therefore, p-hydroxybenzoic acid, which forms the least stable complex with Al, does not inhibit the crystallization of Al hydroxides. On the other hand, aspartic, tannic, malic, and citric acids increasingly retard crystallization (Fig. 1). In addition to the stability constant of the complex, the concentration of the organic acid is important. At certain low concentrations, which vary with the kinds of organic acids, the presence of some organic acids actually promotes the crystallization of particular $Al(OH)_3$ polymorphs, but above the critical concentration, it disrupts crystallization (Huang and Violante 1986). This is because organic acids replace water molecules that would otherwise coordinate with the Al^{3+} ion. The extent to which this occurs depends on the chemical affinity of the organic acid for the Al, i.e., the stability constant, and its concentration relative to Al. Humic substances also influence the transformation of Al by promoting the formation of microcrystalline boehmite and hampering the formation of more crystalline phases (Kodama and Schnitzer 1980; Singer and Huang 1990). Fulvic acids (FA) and humic acid (HA) resemble aliphatic acids, such as citric and malic acids, in that they contain COOH and aliphatic OH groups. They also resemble tannic acid and quercetin, because they contain phenolic hydroxyl and ketonic C=O groups. Through these functional groups, FA and HA form stable complexes with Al and inhibit the crystallization of Al hydroxides. Through perturbation of crystallization, organic substances have a significant impact on the surface properties of Al transformation products. The presence of organic acids during aging of Al hydroxide gels increases the specific surface of the products up to 30-fold over that of the control, and higher organic acid concentrations result in higher specific surface (Kwong and Huang 1978, 1981). The surface charge characteristics of the products also dramatically change. These intermediate transformation products are the most reactive Al species in influencing physical, chemical, and biological processes of soils and associated environments (Huang et al. 2002).

Table 1. Stability constants of the complexes formed between Al and five organic acids at 25°C (Kwong and Huang 1979a)

Organic acids	Stability constants of the complexes	
	log *K*1	log *K*2
p-hydroxybenzoic acid	1.66	–
Aspartic acid	2.60	–
Tannic acid	3.78	–
Malic acid	5.14	8.52
Citric acid	7.37	13.90

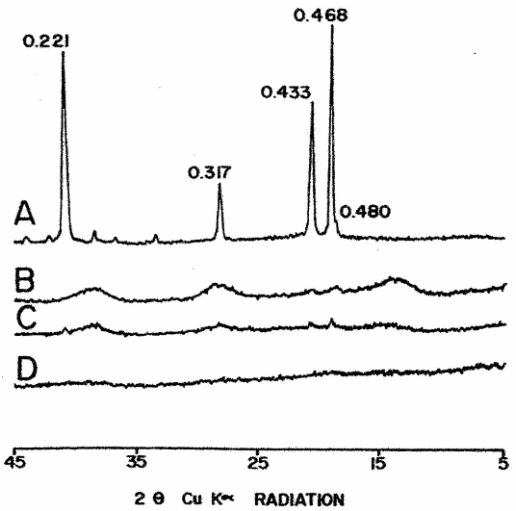

Fig. 1. The x-ray diffraction patterns of hydrolytic precipitation products of Al, showing how four different organic acids influence the transformation to more crystalline phases. The initial Al concentration was 1.1×10^{-3}M at an OH/Al molar ratio of 3 and the solution was aged for 40 d at room temperature in the presence of 10^{-4}M organic acid (Kwong and Huang 1979b).

Organic substances also play a very important role in the formation and transformation of Fe oxides in soils (Fig. 2). In soil environments where the amount of organic matter is low, the Fe supplied will form goethite and hematite depending on environmental factors (Schwertmann 1985). As the organic matter content increases, more of the Fe will be complexed with organics resulting in the decrease in Fe activity. The activity of Fe(III) ions is so low that only the solubility product of goethite (10^{-41}–10^{-42}), but not the solubility product of ferrihydrite (10^{-37}–10^{-39}), is exceeded. Consequently, goethite but not the ferrihydrite may form.

Therefore, no hematite will form in an environment where the organic matter is high, since ferrihydrite is deemed a necessary precursor for hematite. This trend is observed generally in soils in the temperate and cool regions as well as in wet depression and surface soils of the subtropical and tropical regions. At a higher content of organic matter, the rate of Fe supply is high and ferrihydrite will form and may survive for pedogenic times. If the content of organic matter is even higher, such as occurs in O horizons or in peaty environments, all of the Fe may be in the form of Fe-organic complexes. The interaction of organic matter with Fe, thus, plays a vital role in influencing the crystallization and speciation of Fe oxides in soil environments (Schwertmann et al. 1986; Cornell and Schwertmann 1996). Furthermore, the fine scale morphology, mean surface roughness, fractal dimension, specific surface, and microporosity of the Fe oxides depend on the concentration of low-molecular-weight organic acids, e.g., citric acid in the solution in which the Fe oxides are formed (Liu and Huang 1999). Surface properties of Fe oxides formed under the influence of organic substances deserve close attention in advancing our understanding of their surface chemistry pertaining to dynamics and transformations of chemical and biological components in soil and related environments (Huang 2004).

3 Influences of Mineral Colloids on Soil Organic Matter Stabilization and Turnover

Soil minerals play a stabilizing role in organic matter. The Al and Fe that complex and stabilize organic matter against microbial decomposition are released from soil minerals during soil formation. The supply rates apparently control the content of soil organic matter to a great extent. This is demonstrated by the relationship between pyrophosphate-extractable C and pyrophosphate-extractable Al plus Fe (Wada 1995).

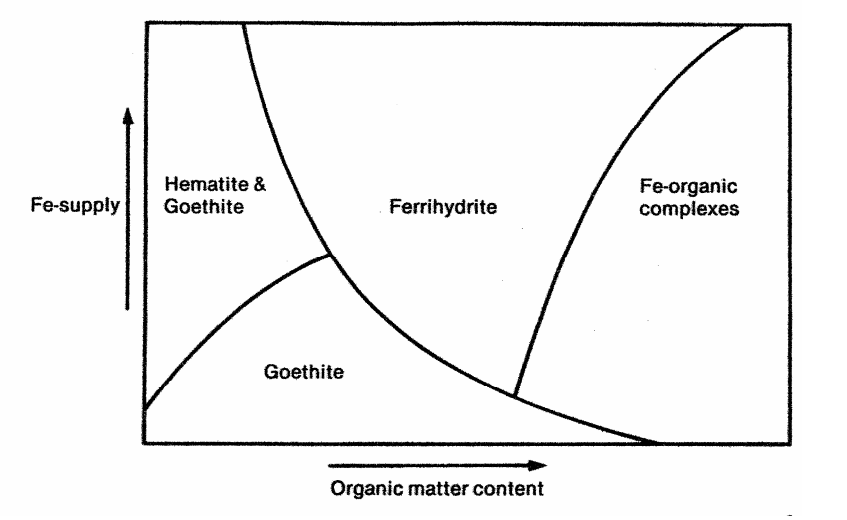

Fig. 2. Tentative schematic representation of the effect of organic matter content and rate of soluble Fe supply on the formation of various Fe forms in soils (Schwertmann et al. 1986).

Torn et al. (1997) investigated interactions between mineralogy and organic C along two natural gradients – of age and of climate – in volcanic soil environments. The total stock of organic C in soil increased with substrate age up to 150 kyr when it was 60 kg m^{-2}, and then decreased to 31 kg m^{-2} over the next four million years (Fig. 3a). Most of the decrease in soil organic C stored in older substrates is attributed to an increase in the turnover of soil organic C, rather than to a decrease in plant productivity. The $\Delta^{14}C$ (‰), which is the turnover time of soil organic matter (i.e., the reciprocal of the decomposition rate), shows that the surface horizons are dominated by fast-cycling organic matter (Fig. 3b). The $\Delta^{14}C$ (‰) values also confirm what is stated above. During the first 150 kyr of soil development, the volcanic parent material weathers to metastable noncrystalline minerals. The amount of noncrystalline minerals increases up to 150 kyr and then declines with greater age (Fig. 3c). In contrast, the amount of more stable crystalline minerals remains low until 150 kyr, then increases steeply (Fig. 3d). Soil organic carbon content follows a similar trend, accumulation to a maximum after 150 kyr, and then decreasing by 50% over the next four millions years (Fig. 3a). The abundance of noncrystalline

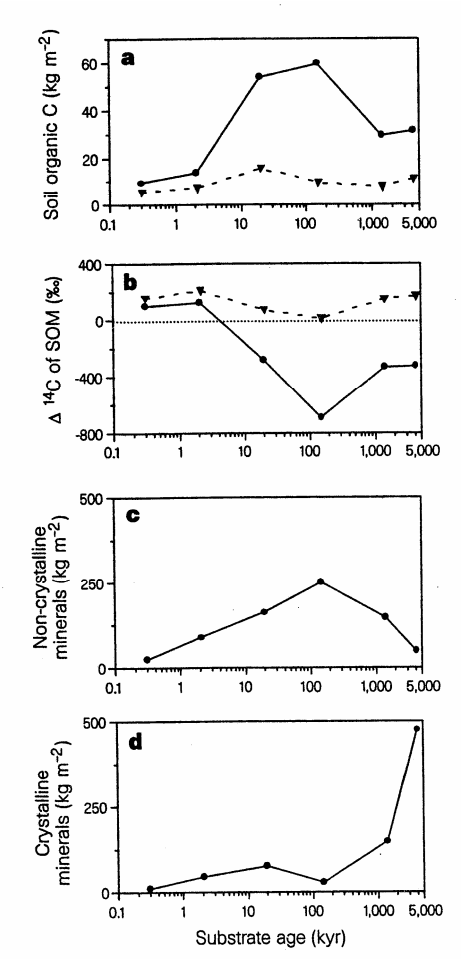

Fig. 3. Soil inventory of carbon in soil organic matter (SOM) **(a)**, $\Delta^{14}C$ of SOM **(b)**, noncrystalline minerals **(c)**, and crystalline minerals **(d)** versus age of soil substrate. Filled circles, total profiles; filled triangle, surface (O and A) horizons (Torn et al. 1997).

minerals accounts for >40% of the variation in organic C content across all the mineral horizons, substrate age, and soil orders. Noncrystalline minerals also strongly influence turnover of soil organic matter. Organic matter $\Delta^{14}C$ is highly and negatively correlated with abundance of noncrystalline minerals (R^2=0.62) except in the oldest site, which has <10% noncrystalline minerals. In contrast, there is no discernible correlation between the abundance of crystalline minerals and C content or turnover time across sites. A positive relationship between noncrystalline minerals and organic

C also exists in soils through the climate gradient. Rasmussen et al. (2005) recently reported significant correlations between Al-humus complexes, and SRO Al mineral species and soil C content, suggesting a chemical protection of organic materials, in addition to the observed physical protection of plant-like material within aggregates. Their results suggest aggregate protection and soil mineral assemblage (namely SRO Al-OH mineral content and Al-humus complex content) significantly control soil C dynamics in the conifer ecosystems. Therefore, soil mineral-organic matter interactions are important in determining the quantity of organic matter stored in soil, its turnover time, and atmosphere-ecosystem carbon fluxes during long-term soil formation.

4 Soil Mineral Catalysis and the Formation of Humic Substances

Humic substances can be synthesized through biotic and abiotic processes. A variety of organic components, such as phenolic compounds, carbohydrates, aldehydes, and nitrogenous substances can participate as starting materials. Soil minerals have the ability to catalyze the abiotic polymerization of phenolic compounds and the polycondensation of phenolic compounds and amino acids and the subsequent formation of humic substances. Kumada and Kato (1970), Wang and Li (1977), and Filip et al. (1977) are among the pioneers in the study of the catalysis of layer silicates on abiotic formation of humic substances through oxidative polymerization of phenolic compounds. Since the early 1980s, Huang and co-workers have studied the sequence of catalytic power of layer silicates and the reaction sites in the polymerization of phenolic compounds and the subsequent formation of humic substances (Shindo and Huang 1985a; Wang and Huang 1986, 1988). Among metal oxides, hydroxides, and oxyhydroxides, Mn oxides are the most powerful catalysts in the transformation of phenolic compounds (Shindo and Huang 1982, 1984). Manganese oxides, which are common in soils, act as Lewis acids that accept electrons from phenolic compounds, leading to their formation of semiquinone and their oxidative polymerization and formation of humic substances. Poorly crystalline aluminosilicates, such as allophane, are common in soils. Allophane has the ability to catalyze the polymerization of polyphenols (Kyuma and Kawaguchi 1964). Even primary minerals have the ability to catalyze the abiotic polymerization of hydroquinone which is a phenolic compound. The sequence of the catalytic power of primary minerals is tephroite > actinolite > hornblende > fayalite > augite > biotite > muscovite ≈ albite ≈ orthoclase ≈ microcline ≈ quartz (Shindo and Huang 1985b).

The Maillard reaction (Maillard 1913) is perceived to be a major pathway in humification because of significant similarities between humic substances and melanoidins (often synthesised *de novo* in microbial cell walls) formed through this pathway involving sugar-amino acid condensations (Ikan et al. 1996). Evershed et al. (1997) reported the presence of characteristic products of the Maillard reaction (alkyl pyrazines) in archaeological plant remains up to 1500 years from Egypt. In spite of the significance of the Maillard reaction, the rates and mechanisms of polycondensation of sugars and amino compounds in nature remains obscure (Ikan et al. 1996). Jokic et al. (2001) reported that birnessite (δ-MnO_2) significantly increases the extent of humification of the glucose-glycine system over the pH range of 6–8 (Fig. 4). The chemical shifts of FA formed in the Maillard reaction systems (Jokic et al. 2001) resemble those of natural humic substances such as soil and stream FAs (Malcolm 1989; Schnitzer 2000). In nature, it is most likely that the polyphenol and Maillard reaction pathways do not occur separately but rather interact with each other. Jokic et al. (2004a) reported that δ-MnO_2, a ubiquitous soil mineral, significantly accelerates humification processes in a system containing glucose, glycine, and catechol at temperatures and pH typical of natural environments. Their data indicate the signicance of linking the polyphenol and Maillard reactions, as promoted by mineral colloids such as δ-MnO_2, into an integrated humification pathway.

5 Interactions of Enzymes with Soil Mineral and Humic Colloids and Impacts on Enzymatic Activity

Extracellular enzymes are rapidly sorbed at mineral and humic colloids in soils and sediments. Mineral colloids have a high affinity for enzymes although that is not always synonymous with the retention of their catalytic ability. On the other hand, humic substances have the ability to sorb and sequester enzymes in such a way as to retain their catalytic activity; they could also strongly inactivate enzyme activity depending on interaction mechanisms.

Mineral colloid-enzyme interactions have been documented (e.g., Theng 1979; Burns 1986; Naidja et al. 2000; Burns and Dick 2002). Besides cation-exchange reactions, adsorption of enzymes by mineral colloids may proceed through ionic, covalent, hydrophobic, hydrogen bonding, and van der Waals forces. When enzymes are adsorbed on mineral colloids, changes in the tertiary structures (i.e., the folding of the helix or

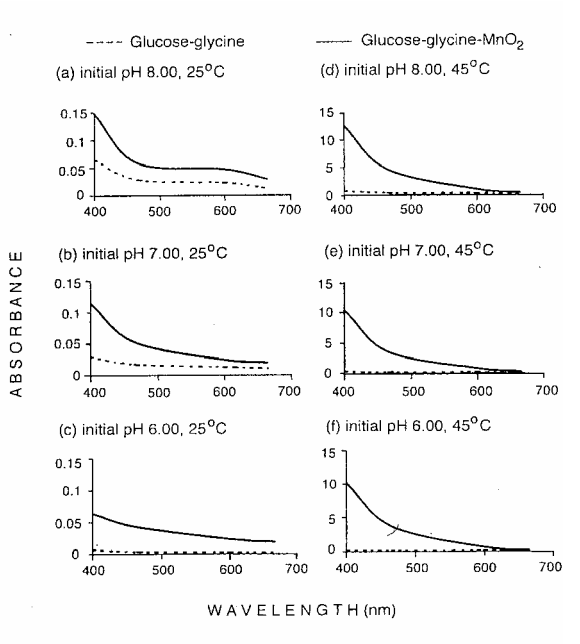

Fig. 4. Absorbance versus wavelength plots in the Maillard reaction between glucose and glycine as influenced by birnessite catalysis. **(a)**, **(b)**, and **(c)**: 30 d reaction period. **(d)**, **(e)**, and **(f)**: 15 d reaction period (Jokic et al. 2001).

coil into a compact, globular molecule stabilized by interfold hydrogen bonding, van der Waals, and hydrophobic interactions) of the enzymes and their active sites decrease the activity of the enzyme or eliminate it altogether (Burns 1986). However, there are notable exceptions to the adsorption-decline in activity rule. Various supports differ in their ability to immobilize enzymes (Table 2). The residual specific activities of laccase and peroxidase immobilized on and/or within all supports including glass beads, montmorillonite, kaolinite, and soil are high. Furthermore, laccase immobilized on montmorillonite shows specific activities that are higher than those of the free enzyme (Table 2). This may be attributed to steric modification of the immobilized enzymes such that the active site becomes more exposed to substrates. Although mineral sorption often stabilizes enzymes against degradation (Tietjen and Wetzel 2003; Kelleher et al. 2004), mineral-bound compounds are unable to diffuse, thereby reducing the encounter rates between enzymes and substrates. Even if some substrates do diffuse to bound enzymes, the active sites may be partially blocked so that enzymatic catalysis is reduced, as evidenced by reductions in the activity of some mineral-sorbed enzymes (Gianfreda et al. 1992). Conversely,

mineral-bound C substrates may be physically prevented from entering the active sites of mobile enzymes (Sollins et al. 1996). In addition to the large surface areas of mineral colloids that facilitate sorption, many mineral colloids also contain micropores (Yu et al. 2006) or mesopores or physical structures that can help trap small organic compounds and exclude degradative enzymes (Mayer et al. 2004; Zimmerman et al. 2004a,b). Furthermore, in extreme cases, substrates, enzymes, and microbes may all be present in soils but so strongly bound to mineral surfaces and physically protected that substrate degradation is minimal and enzymatic products are unlikely to reach microbes (Allison 2006a). This scenario may help to explain why the C associated with reactive mineral colloids can be tens of thousands of years old (Torn et al. 1997). The performance of enzymes in the terrestrial ecosystem is, thus, substantially influenced by mineral colloids. The role of SRO oxides, hydroxides, and oxyhydroxides of Al, Fe, and Mn (as well as non soil supports) in influencing enzymatic activity pertaining to the transformation of natural and anthropogenic organics merits increasing attention (Huang 1990, 2004; Naidja et al. 2000; Violante and Gianfreda 2000).

Table 2. Immobilization of a laccase (from *Trametes versicolor*) and a peroxidase (from horseradish) on different supports (Gianfreda and Bollag 1994)

Enzyme and support	Enzymatic activity			
	Protein adsorbed[a] (mg/%)	Units adsorbed[b]	Specific activity[c]	Residual specific activity[d]
Laccase				
Glass beads	0.452/56	28.8	63.7	236
Montmorillonite	0.622/71	19.8	31.8	118
Kaolinite	0.566/64	13.1	23.1	85.5
Soil	0.644/73	15.7	24.4	90.4
Peroxidase				
Glass beads	0.092/17	8.4	91.6	93.8
Montmorillonite	0.224/43	23	102.8	105.2
Kaolinite	0.120/23	9.5	78.9	80.7
Soil	0.162/31	15	92.6	94.8

[a] Difference between proteins initially added to 200 mg of support (0.88 mg laccase and 0.52 mg of peroxidase) and those recovered in the supernatant and washings.
[b] Expressed as μmol O_2 consumed min^{-1} for laccase and μmol guaiacol transformed min^{-1} for peroxidase.
[c] Units adsorbed/protein adsorbed
[d] Calculated as percentage of the specific activity (sa) of the free enzyme (laccase, sa = 27 μmol min^{-1} mg^{-1}; peroxidase, sa = 97.7 μmol min^{-1} mg^{-1}).

It has been reported that soil organic matter can inhibit enzymes (Vuorinen and Saharinen 1996) and enzyme activity may be reduced by adsorption on humic polymers (Gianfreda et al. 1998). Enzyme inhibition by humic substances has been well demonstrated for an oxidoreductase (Pflug 1980; Sarkar and Bollag 1987), a protease (Ladd and Butler 1969), an invertase and a phosphatase (Malcolm and Vaughan 1979). On the other hand, Kang et al. (2002) and Park et al. (2000) reported that, although high concentrations of humic-like polymers tend to inhibit laccase-mediated transformation of xenobiotics (including catechol), low concentrations of humic acid might enhance the enzymatic transformation of phenolic compounds. Furthermore, it has been reported that enzymes can be stabilized against all sorts of impacts (e.g. temperature, solvents, pH, proteases) by soil organic matter (Conrad 1942; Burns 1986; Nannipieri and Gianfreda 1999). Mechanisms proposed to account for the stability of enzymes by soil organic matter include ion exchange, H-bonding, covalent bonding, lipophylic reactions, and entrapment within three-dimensional micelles during organic matter genesis. Enzyme-humic complexes can also be bound to mineral colloids and this may further enhance enzyme stability.

Recent research data of Allison (2006b) suggest that enzyme activity measured in the laboratory represents the sum of active and stabilized enzyme pools. Common soil minerals such as allophane and ferrihydrite, partially determine the size of the stabilized pool. In contrast, humic acid, which is among the most abundant organic components in soil, strongly inactivate enzyme activity, although enzymes incorporated into humics during humic polymer synthesis may be more stable. The functional importance of stabilized enzymes still remains uncertain, and evidence from the literature suggests that the active enzyme pool is more strongly associated with biogeochemical process (Allison 2006b). Future research should address the relative contributions of different enzyme pools to ecosystem function (e.g., Stemmer et al. 1999). Studies measuring bulk enzyme activities in soil should recognize that a large pool of stabilized enzymes could make changes in the active pool more difficult to detect. Compared with bulk soil enzymes, active enzymes probably correlate more closely with soil quality mineralization rates, or disturbance. Therefore, ecosystem models should incorporate multiple pools of enzymes to improve predictions of organic matter decomposition, especially if stabilized enzymes have reduced catalytic efficiency.

6 Influence of Mineral Colloids on the Structure, Dynamics, and Activities of Microbial Communities

Soil is a habitat for myriads of microbes. The microbial biomass constitutes only a very small proportion (<3% of the total organic C in soil). However, it is the most active and dynamic fraction of the living organic pool. Mineral colloids are the most reactive fraction of inorganic components of soils because of their large specific surface areas and high charge density characteristics. Being enriched in ions, water, and organic matter relative to the bulk soil, the surface of mineral colloids serves as a preferred habitat for soil microbes (Theng and Orchard 1995).

The surface of bacterial cells and crystalline clay minerals are both negatively charged. However, bacteria have the propensity for producing extracellular polysaccharides (EPS) which bind simultaneously to cell and clay surfaces through cation bridging involving polyvalent cations (Fig. 5). EPS production aids the retention of bacterial cells within comparatively active biofilm communities at clay (or root) surfaces (Lunsdorf et al. 2000). The predominantly negatively charged mineral colloids in soils are largely coated with hydroxy Al (or Fe) polymers. As a consequence, these coated minerals behave as a positively charged species or display amphoteric characteristics. Therefore, mineral colloids can strongly interact with negatively charged microbial cells in soils. This type of chemical bonding, which is much stronger than cation bridging, is also expected to occur with Al and Fe oxides over the pH range of soils. Microbial cell wall charge characteristics are indeed pH dependent according to the dissociation constants of their exposed cell wall functional groups. The attachment of microbes to SRO mineral colloids and the crystal edges of layer silicates through electrostatic interactions would also be predicted to occur when the soil pH falls below 6 because the net charge of all of these mineral surfaces would then be positive and the surface of all bacteria and fungi would be virtually negatively charged (Theng and Orchard 1995).

In the majority of cases, minerals in topsoils are partially covered with organic materials, especially humic substances, which are to a large extent microbially resistant. The most common mode of mineral colloid-organic material-microorganism interactions may be depicted as follows (Theng and Orchard 1995):

$$[\text{Mineral colloid}—\text{HS}]^{-} \ldots \text{M}^{n+} \ldots [\text{EPS-B}]$$

where HS is humic substances, M is divalent/polyvalent metal cation, EPS extracellular polysaccarides, and B microbes and/or biofilm.

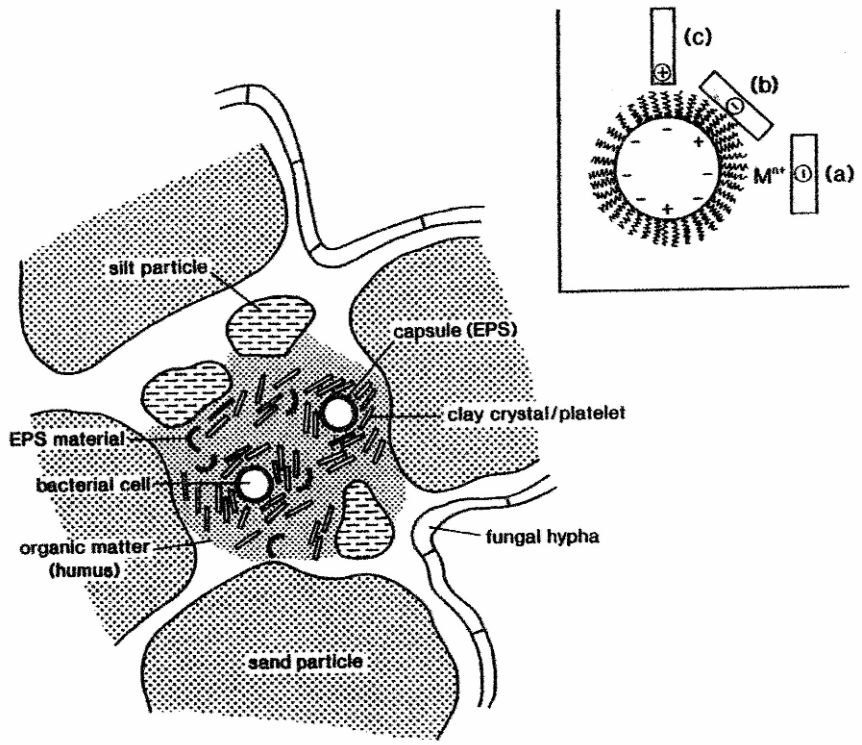

Fig. 5. Diagram illustrating the interaction of bacteria and fungi with mineral particles in a soil aggregate (Theng and Orchard 1995). Bacterial cells with a coat of extracellular polysaccharides (EPS) are enveloped by clay particles. The pore space where clays and bacteria interact, bounded by silt- and sand-size particles, is relatively enriched in organic matter including EPS residues. Fungal hyphae are attached to the outside surface of an aggregate. Inset shows an enlarged view of a bacterial cell with its complement of EPS. At normal soil pH conditions, the cell has a net negative surface charge. Most clay particles adhere to the cell surface by bridging through polyvalent cations, represented by M^{n+} (**a**) although some may be attached directly by electrostatic interactions, either in face-to-face (**b**), or edge-to-face (**c**) association.

In humic-rich calcareous Mollisols, Ca would be the predominant bridging cation. In Andisols, Oxisols, Ultisols and the B horizon of Spodosols, HS largely occur as complexes with Al and Fe, or their respective SRO oxides (Theng et al. 1989; Oades et al. 1989). In soils with little organic matter and in subsoils, mineral colloid-microorganism interactions are largely influenced by the mineralogical composition and pH of the soil. Besides the existing literature (Stotzky 1986, 2002; Theng and Orchard 1995; Huang and Bollag 1999; Huang 2004; Huang et al. 2005) much

more research is needed to further understand the mechanisms of surface interactions of mineral colloids with microorganisms.

Mineral colloids can directly or indirectly influence the activity of microorganisms in their immediate vicinity (Stotzky and Burns 1982; Stotzky 1986). The effect of mineral colloids may be positive, negative, or sometimes so small as to escape detection. Mineral colloids have a stimulating effect on the activity of adhering bacteria by keeping the pH of microhabitats within the optimum physiological range for growth. The content and type of mineral colloids are influential in determining the balance between microbial populations in soil. A well-known example is the failure of some fungi to thrive and spread in certain soils (Stotzky 1986). This is largely attributed to the presence of montmorillonite in the suppressive soils. Montmorillonite can serve as a proton sink and is thus able to promote the growth of acid-sensitive bacteria in these soils. This gives bacteria a selective advantage over fungi in competing for available nutrients. Fungal growth and proliferation are, thus, effectively suppressed.

Microbial activity can also be stimulated by mineral colloids through their ability to sorb metabolites that would otherwise have an adverse effect on microbial growth (Filip et al. 1972; Filip and Hattori 1984) This may be due to the toxicity of metabolites, and their feed back repression and, encouraging competitors. Predictably, montmorillonite (CEC ~100 cmol kg^{-1} and specific surface of ~800 m g^{-1}) is more effective than kaolinite and finely ground quarts. Other substances, such as antibiotics and pesticides that are toxic to some microorganisms, can also be adsorbed by the surfaces of mineral colloids (Theng and Orchard 1995; Dec et al. 2002).

Furthermore, adhering microorganisms may benefit from being close to nutrients adsorbed on the surface of mineral colloids (including those concentrated in a cloud in the diffuse double layer). However, the potential substrates may not be readily available or physically accessible (Fletcher 1991) especially if intercalated. Moreover, the addition of mineral colloids to the system beyond a certain concentration may result in a reduction in microbial activity due to restricted diffusion of oxygen and nutrients to microbial cells (Marshall 1971). This is attributable to the progressive enveloping of microbial cells by minerals colloids. Timmis and his co-workers reported a novel interaction between bacteria and clay minerals (Lunsdorf et al. 2000). The biofilms that developed consist of a dense lawn of clay aggregates, each one of which contains one or more bacteria, phyllosilicates, and grains of iron oxides, all held together by bacterial EPS. The clay leaflets are arranged in the form of ‘houses of cards’ and give the aggregates the appearance of ‘hutches’ housing the bacteria. The ‘clay hutches’ may represent a ‘soil microhabit’, a ‘mineral nutrition sphere’,

and an 'effective survival unit' for autochthonous bacteria. The formation of composite biofilms and clay hutches through interactions of bacteria and mineral colloids merit investigation, particularly regarding microbio-geological processes (Lunsdorf et al. 2000, 2001).

Mineral colloids, by forming an envelope around bacterial cells, may provide protection from extreme fluctuations in physicochemical environments and thus, enhance bacterial survival (Stotzky 1986; Theng and Orchard 1995). The protective effect of mineral colloids, especially montmorillonite, is manifested in the ability of soil microbes to withstand exposure to hypertonic osmotic pressure, desiccation, and ultraviolet radiation. Besides serving as a substratum for bacteria to adhere to and as a protective envelope of bacterial cells, mineral colloids can act as a cementing agent of soil particles to influence aggregation. The addition of clay to soils, especially those of light texture would, thus, modify the spatial arrangement of particles and the pore-size distribution within aggregates. Such a modification of aggregate structure often benefits the bacterial population by increasing the proportion of pores of a certain size range (< 6 μm) from which bacteria could freely enter to colonize pores but bacterial predators notably protozoa are effectively barred from entering such pores due to steric hindrance. More recently, it has been reported that microbial activity is influenced by mechanical limits (Rebata-Landa and Santamarina 2006). Pore and pore-throat sizes may restrict habitable pore space and traversable interconnected porosity. Soil- or sediment-cell interactions may cause puncture or tensile failure of the cell membrane. Soil structure largely determines energy flows and the distribution and composition of soil microhabitats (Mummey and Stahl 2004).

7 Microbial Mediation of Weathering Transformation of Soil Minerals and Metal Dynamics

Chemical weathering of minerals during pedogenesis can be enhanced by microbial activity by a factor as high as 10^6 (Kurek 2002). Microorganisms can dissolve minerals by direct and indirect actions under aerobic and anaerobic conditions (Robert and Berthelin 1986; Ehrlich 2002; Kurek 2002). In some cases of attack, the microorganisms may be dispersed in the soil solution; in others, they may grow in biofilms on the surface of susceptible minerals.

Oxidation by chemolithotropic microorganisms of the sulfur entities of metal sulfides to obtain energy is an example of direct dissolving action under aerobic condition (Kurek 2002). Compounds of many other

oxidized metals and metalloids such as Fe(III), Mn(IV), and As(V) can act as electron acceptors in anaerobic environments. Under such conditions, anaerobic respiration becomes an example of direct dissolving action of oxidized metal and metalloid compounds.

Fungi can adsorb K from solution and thus shift K equilibrium in suspension of trioctahedral and dioctahedral micas and transfer them to vermiculites (Weed et al. 1969). Similar processes can also occur for many major and trace elements (Robert and Berthelin 1986). Because of their small size, microorganisms provide a large contact area that interact with metals in the environment. Microbial metal accumulation has recently received much attention particularly due to the potential use of microorganisms for cleaning metal-polluted water. (Sag 2001; Fomina and Gadd 2003; Arica and Bayramolgu 2005; Bishnoi and Garima 2005; Kahraman et al. 2005; Pal et al. 2006). The different accumulation processes that microorganisms perform have been analyzed and their potential significance in soil systems has been addressed (Ledin 2000). Different mechanisms can be involved in the accumulation of metals by microorganisms, e.g., adsorption, precipitation, complexation, and active transport into the cell. Physicochemical parameters such as pH, ionic strength, ionic composition as well as biochemical and biological factors are of importance in influencing the magnitude of accumulation. Several recent studies have applied surface complexation theory to model metal adsorption behavior onto microorganisms (e.g. Burnett et al. 2006). Surface complexation models (incooperating the Dorman electrostatic model) have been developed to determine stability constants corresponding to specific adsorption reactions. Adsorption reactions and stoichiometries have been constrained using spectroscopies such as attenuated total reflectance FTIR (ATRFTIR), x-ray absorption near edge structure (XANES) and extended x-ray absorption fine structure (EXAFS). Molecular simulations of metal adsorption to microbial surfaces have recently been reported; force field-based simulation techniques can adequately describe the interactions of Cd with the cell wall, defining both metal ion coordinations and binding distances (Johnson et al. 2006). These research findings further indicate that microorganisms should also accumulate metals in soils and the amounts accumulated may be considerable. Therefore much work remains to be done, with focus on mechanisms of microbial accumulation of metals in soils. Considerable less attention has been paid to the role of microorganisms in metal mobility. It is, thus, important to determine the overall influence of soil microorganisms on metal accumulation and mobility and to quantify these processes.

Mineral diagenesis, which is the transformation of one mineral into another by some microorganisms, can be an indirect effect of aerobic and anaerobic microbial metabolism (Ehrlich 2002; Kurek 2002). The

formation of a new mineral can be resulted from a chemical reaction between products of microbial dissolution of a mineral in the environment.

The physical and chemical characteristics of bacteria, such as their large surface area-to-volume ratio, serve to increase the metal-binding capacity of their charged surfaces leading to precipitation and formation of mineral phases on their cell walls or other surface polymers (McLean et al. 2002). Therefore, geochemical modeling of metal speciation and transport is beginning to include bacteria as geochemically active surfaces (Huang and Bollag 1999; Burnett et al. 2006). The mechanisms by which bacteria initiate the formation of minerals in bulk solution vary widely between species. There may be a combination of biochemical and surface-mediated reactions during the process.

The formation of Mn oxides is an example of microbially mediated fine-grained mineral development. Microbial oxidation of Mn(II) is a major process that can produce Mn oxide coatings on soil particles 10^5 times faster than abiotic oxidation (Tebo et al. 1997). This microbially mediated formation is illustrated in Fig. 6. Manganese oxides are highly reactive minerals and help restrict the mobility of metals in soils and sediments through adsorption on their surfaces. Biogenic Mn oxides formed by *Leptothrix discophora* SS-1 have significantly larger specific surface and higher Pb adsorption capacity than abiotically precipitated Mn oxides (Nelson et al. 1999). Bioformation of minerals should, thus, have a significant bearing on restoration of metal-contaminated soils.

8 Mineral Colloid-Organic Substance-Microorganism Interactions in Relation to Soil Structural Stability

Root exudation and microbial action produce organic compounds with a range of composition and molecular weights. These compounds interact with the mineral particles, which also vary in size, shape, crystallinity, and electric charge (Emerson et al. 1986). Interactions between soil mineral particles, organic matter and microbes can occur at many different size scales, because these materials have a large size range in soils (Fig. 7).

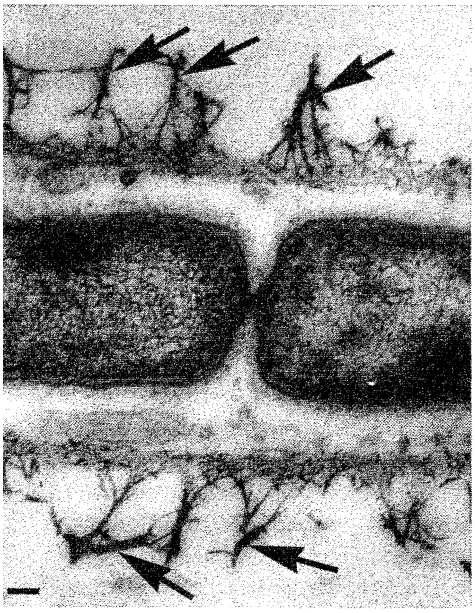

Fig. 6. Thin section of *Leptothrix* sp. which is precipitating manganese oxide on its outermost structure called a sheath (McLean et al. 2002). The arrows point to the manganese mineral phase identified by EDS. Scale bar = 150 nm.

Organic molecules such as microbially derived polysaccharides and other unaltered and altered biomolecules can be adsorbed on mineral surfaces, resulting in enhancing the stability of individual clay microstructures. Adsorption is essential in binding together clay microstructures and silt particles into small microaggregates with 2–50 μm diameters and densities > 2.0 Mg m^{-3} (Baldock 2002). Many microaggregates exist as pieces of fungal hyphae, bacteria or bacterial colonies coated with EPS and clay minerals (Oades and Waters 1991). Particulate organic matter (POM) are essential stabilizing agents at larger size scales, i.e., large microaggregates and small macroaggregates (Jastrow and Miller 1998). Soil structure can be stabilized by POM through two mechanisms related to its physical properties and its susceptibility to biological decomposition (Baldock 2002). POM can bridge the failure zones that exist between adjacent stable aggregates; it can also enhance the stability of soil structure by providing a substrate for microorganisms to enhance the production of fungal hyphae and microbial metabolites such as polysaccharides. While POM continues to provide a substrate for microorganisms, the production of biochemical aggregating agents continues, and the structural stability is maintained. Glomalin is a recently discovered glycoproteinaceous substance produced

by arbuscular mycorrhizal fungi (AMF). Wright and colleagues showed the relationship between the concentration of glomalin in soil and the stability of aggregates under different crops or cropping systems (Wright et al. 1999; Wright and Anderson 2000; Franzluebbers et al. 2000). The concentrations of glomalin-related soil protein (GRSP) are positively correlated with aggregate water stability and GRSP has a relatively slow turnover in soil (Rillig 2004; Driver et al. 2005). The discovery of a strong affinity of glomalin for mineral particle suggests that the role of AMF hyphal systems in the formation of microaggregates needs further investigation (Goss and Kay 2005). The influence of mycorrhizas on key ecosystem process of soil aggregation warrants in-depth study (Rillig and Mummney 2006).

Since organic matter responsible for the stabilization of soil structure is not inert and thus subject to decomposition, aggregation is a dynamic process in soils (Baldock 2002). Biological activity has the potential not only to stabilize soil structure through the production of organic substances capable of binding soil particles, but also to destabilize soil structure by decomposing organic binding agents. The balance between these two processes dictates the level of soil structural stability. Therefore, a continuous input of organic materials, mainly from plant production is essential to sustain or enhance soil structural stability.

9 Biogeochemical Transformation and Transport of Nutrients and Pollutants

In general, more than 95% of the N and S and between 20 and 90% of the P in surface soils are present in soil organic matter (Guggenberger and Haider 2002). The close relationship between the organic forms of C, N, P, and S is well established. The turnover of organic C is closely associated with the dynamics of N, P, and S in soils. Soil mineral colloids have a profound influence on the stabilization and degradation of soil organic matter and its associated nutrients. Chemical and physical interactions of minerals with soil organic matter result predominantly in the stabilization of the organic matter. Recently, it has been shown that the promoting action of Mn(IV) oxide on the Maillard reaction (sugar-amino acid condensation) under ambient conditions results in the abiotic formation of heterocyclic N compounds, which are often referred to as *unknown* N and of amides which are apparently the dominant N moieties in nature (Fig. 8).

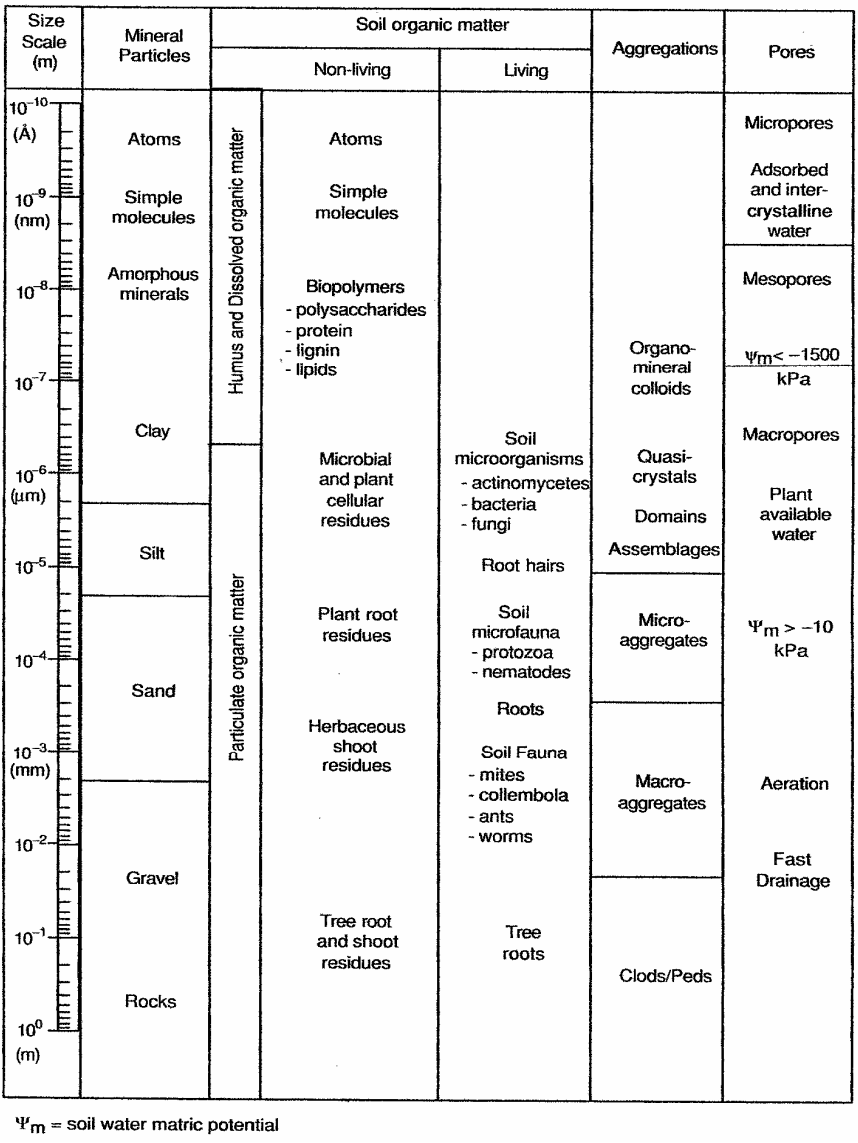

Fig. 7. Size scale associated with soil mineral particles, organic components, pores and aggregations of mineral and organic components (Baldock 2002). The definitions of pore size have used those developed by IUPAC (micropores < 2 nm, mesopores 2–50 nm and macropores > 50 nm). Alternatively, the pore sizes corresponding to the lower (ψ_m = – 1500 kPa) and upper (ψ_m = – 100 kPa) limits of water availability to plants may be used to define the boundaries between the different classes of pore size. ψ_m is soil water metric potential.

Organic matter may weather soil minerals (Robert and Berthelin 1986) and impede crystallization of secondary minerals (Schwertmann et al. 1986; Huang et al. 2002). Noncrystalline minerals have the ability to stabilize soil organic matter and reduce the turnover of C, N, P, and S (Torn et al. 1997; Guggenberger and Haider 2002). Organic matter also acts as a binding agent to promote aggregation, which, in turn, reduces the turnover of these nutrients through occlusion by minerals. There are distinct interactive mechanisms between soil minerals and organic matter, which have direct effects on the turnover and cycling of C, N, P, and S.

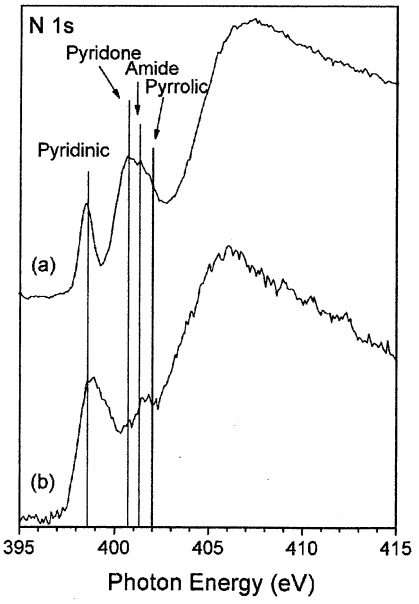

Fig. 8. N Is XANES spectra of **(a)** fulvic acid isolated from a glucose-glycine-δ-MnO_2 system and **(b)** the lyophilized solid phase (Jokic et al. 2004b). The peaks are assigned to pyridinic (398.6 eV), pyridone (400.7 eV), amide (401.3 eV) and pyrrolic (402.0 eV) moieties.

Both enzymes and mineral colloids are involved in catalytic transformations of organic pollutants in soils. Although microbial inhibitors can be used to quench biotic processes, their side effects on the surface reactivity of mineral colloids remain uncertain. Therefore, it is generally difficult to determine whether an organic pollutant is transformed abiotically or biotically (Huang 1990) but both may take place simultaneously (Huang and Bollag 1999). The degradation of organic pollutants by extracellular enzymes is well documented (Dec et al. 2002). Enzymes commonly occurring

in soil, such as esterases, amidases, phosphatases, and proteases catalyze the hydrolysis of the respective chemical bonds in xenobiotic molecules. On the other hand, extracellular phenoloxidases have the ability to catalyze the transformation of phenolic or anilinic compounds to their polymerized products (Bollag 1992) thereby reducing their bioavailability. They are implicated in binding of xenobiotics to soil by catalysis of the oxidation of organic pollutants to free radicals, followed by chemical coupling of oxidation products to humic substances. Enzymes are present either free in solution or bound onto mineral colloids, humic substances, and mineral colloid-humic complexes (Burns 1986). This immobilization is the major factor that determines their survival and performance in soil environments. Therefore, immobilization of enzymes may have a considerable impact on the rate of xenobiotic degradation (Dec et al. 2002) and have significant applications in bioremediation (Bollag et al. 2005). After immobilization, enzymes show generally increased stability toward physical, chemical, and biological denaturation (Ruggiero et al. 1996; Naidja et al. 2000). However, immobilization may have a considerable effect on the activity and kinetic behavior of enzymes in the transformation of organic pollutants due to steric and diffusional restrictions, direct involvement of the active sites in binding to the support, and modified conformation of the immobilized enzymes (Matinek and Mozhaev 1985; Quiquampoix 1987; Quiquampoix et al. 1995, 2002; Naidja et al. 2000, 2002). Conformational alteration of enzyme molecules may result in the decrease in accessibility of the active sites to the substrate, causing a setback in substrate transformation. However, there are notable exceptions to this trend (Burns 1986; Gianfreda and Bollag 1994; Naidja et al. 2000). To date, the knowledge on detailed surface structures of the enzyme-mineral colloid and enzyme-humus complexes at the molecular level still needs to be advanced. More research is needed to uncover new enzymes and new mineral composite supports to be used for catalytic degradation of a wide range of industrial and agricultural pollutants in soil and related environments.

Most of the organic chemicals, including xenobiotics, exhibit a strong affinity to humic substances. However, the transformation of xenobiotics in terrestrial systems is greatly influenced by mineral components of soil (Huang 1995). Mineral colloids, which are present in high concentration in soil and have large specific surface and relatively high charge density, contribute to the overall xenobiotic transformation at least as much as does the organic matter. Organic matter can induce surface-catalyzed reactions of adsorbed pesticides, but it could hinder the degradation of some pesticides by decreasing both their availability to microbial attack and their concentration in the soil solution (Huang and Bollag 1999). The significance of soil mineral-catalyzed abiotic transformation of

xenobiotics in the environment has also become widely recognized. The processes of adsorption and abiotic degradation of xenobiotics through the action of the surfaces of soil minerals vary with the structural and surface properties of the minerals, saturating cations and hydration status, molecular structures of xenobiotics, and associated environmental factors.

The degradation of organic pollutants may be considerably reduced when they are retained by soil colloids. The major reason for reduced biodegradation rates is the diminished bioavailability of chemicals involved in binding processes (Alexander 1995). The availability of sorbed xenobiotics to microorganisms varies with the chemical properties of pollutants, the nature of the sorbent, the mechanisms of sorption, and the properties of the degradative organisms (Guerin and Boyd 1992; Dec et al. 2002; Ehlers and Loibner 2006). The mechanism of sorption also may change with residence time of xenobiotics present in soil leading to changes in their bioavailability (Alexander 1995). As soil-pollutant contact time increases, pollutant bioavailability and extractability decreases (Reid et al. 2000; Alexander 2000). This phenomenon has been termed "aging". Decreased chemical extractability with increased soil-chemical contact time is evident where both "hash" techniques (e.g. dichloromethane Soxhlet extraction) and "non-exhaustive" techniques (e.g. butanol shake extraction) have been used. Similarly, decreases in bioavailability with increased soil-pollutant contact time have been described in bacterial, earthworm, and other organism studies. Furthermore, the fraction of pollutant determined to be bioavailable can vary between organisms. Thus, there is an immediate definition problem, what is bioavailability? If bioavailability is to be assessed by a chemical means, which organisms should (or can) be mimicked by the extraction? Bioavailability is an important consideration in risk assessment of soil contaminants and in the selection of appropriate remediation techniques for polluted sites (Braida et al. 2004; Stokes et al. 2005; Ehlers and Loibner 2006). Future work should emphasize the biological significance of bound residues and their release (Barraclough et al. 2005).

Microorganisms are closely associated with solid surfaces in surface soils. Sorption of microorganisms may be extremely extensive, especially in soils with high contents of clay and organic matter. Microorganisms may use many different mechanisms to achieve and maintain attachment (Dec et al. 2002). The effect of adsorption of microorganisms on soil colloids on their ability to degrade organic compounds is difficult to predict. The microbial degradation may be enhanced or decreased, or may remain unchanged as the cells undergo the adsorption to solid surfaces. This is apparently dependent on the nature and properties of solid surfaces and substrates, and the kinds of microorganisms. However, the

utilization of sorbed organic pollutants by microorganisms that adhere to the same surfaces is a common phenomenon (Alexander 1999). The impact of structural configuration and surface properties of soil mineral and organic components on the activity of microorganisms and their ability to degrade organic pollutants with different structure and functionality merits close attention.

The transformation of metals in the environment is influenced by interactions of physicochemical, biochemical, and biological processes (Huang 2008). The impacts of these interactive processes on metal transformation are especially important in the rhizosphere where the kinds and concentrations of substrates are different from those of the bulk soils because of root exudation (McLaughlin et al. 1998; Huang and Germida 2002; Huang and Gobran 2005). This leads to colonization by different populations of bacteria, fungi, protozoa, and nematodes. Plant-microbe interactions result in intense biological processes in the rhizosphere. These interactions, in turn, affect physicochemical reactions in the rhizosphere. The total rhizosphere environment is governed by an interacting trinity of the soil, the plant, and the organism associated with the root (Lynch 1990a,b). Therefore, reactions and processes in the soil rhizosphere, which is the bottleneck of metal contamination of the terrestrial food chain, can be understood satisfactorily through interdisciplinary team research. Much of the research on physicochemical reactions of metal transformation in soil has used well-defined model systems which do not involve physicochemical-biological interactions. The reactions and processes which influence metal transformations include redox reactions, complexation reactions, adsorption-desorption reactions, precipitation-dissolution reactions, uptake by microorganisms, biomineralization, microbial excretions, and mycorrhizal infection (Huang and Germida 2002). The impacts of these physicochemical, biochemical, and biological interactions in soils on metal transformation and bioavailability, food chain contamination, and ecosystem health deserve increasing attention.

10 Summary and Conclusions

Soil is the central organizer of the terrestrial ecosystem. Soil constituents, be they minerals, organic matter, or microorganisms, are of prime importance in governing interactive physical, chemical, and biological processes in soil environments.

These physical-chemical-biological interfacial interactions govern weathering transformations of minerals, storage and turnover of organic

matter, enzymatic activities, the structure, dynamics, and activities of microorganisms, porosity formation and structural stability of aggregates, and biogeochemical transformations and transport of chemical and biological components at different temporal and spatial scales. Fundamental understanding of these interfacial interactions at the molecular level is essential to understanding dynamics and mechanisms of environmental processes and the impacts on ecosystem restoration.

Scientific progress is based ultimately on unification rather than fragmentation of knowledge. The important sub-disciplines of a scientific discipline have been altogether too loosely coupled to the frontier in the first place, and this has compromised their ability to take the next step forward. The time is upon us to recognize that the new frontier is the interface, wherever it remains unexplored (Kafatos and Eisner 2004).

In my view, the interfaces are the most underdeveloped areas of the sciences including Soil Sciences. Fundamental understanding of soil physical-chemical-biological interfacial interactions is a major step forward in advancing our understanding of *in-situ* interactive soil reactions and processes and their impacts on human welfare.

Acknowledgments

The funding provided by Discovery Grant GP 2383 of the Natural Sciences and Engineering Research Council of Canada was greatly appreciated.

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2 Sorption and Desorption of Arsenic by Soil Minerals and Soils in the Presence of Nutrients and Organics

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1 Introduction

Arsenic is an element ubiquitous in the Earth's crust and is extremely toxic for humans, animals and plants. Its occurrence in natural environments may be due to natural processes (weathering reactions, biological activities and volcanic emissions) as well as anthropogenic activities (Matera and Le Hécho 2001; Frankenberger 2002; Mandal and Suzuky 2002; Smedley and Kinniburgh 2002). The mean content of arsenic in soils is of the order of 5–10 mg kg^{-1}. Arsenopyrite (FeAsS) is the most abundant arsenic-containing mineral and other minerals include realgar (AsS), orpiment (As_2S_3), olivenite (Cu_2OHAsO_4). Arsenic-bearing herbicides and pesticides have been widely used in agricultural practice until the mid-1900. In particular, lead arsenates and especially schultenite ($PbHAsO_4$), were used estensively as insecticides in orchard soils (Cancés et al. 2005). As a result

of application of arsenical pesticides to fruit crops in orchard soils concentrations of arsenic in the range 366–732 mg kg^{-1} have been quoted (Ure and Berrow 1982).

Natural contamination of ground waters by arsenic has become a crucial water quality problem in many parts of the world (Berg et al. 2001; Chakrabarti et al. 2002; Smedley and Kinniburgh 2002, and references there in). Arsenic in drinking water is much more bioavailable than arsenic in soil, because water-soluble arsenic is rapidly sorbed by humans (Yang et al. 2003). Recently, the European Union and the USA with National Priorities List (NPL) have fixed a limit of 10 μg As L^{-1} in drinking water. In Bangladesh, over 75% of water used for irrigation came from groundwater. A huge amount of arsenic is thus transferred from the contaminated aquifer to the surface soil-plant system (Smedley and Kinniburgh 2002; Martin et al. 2007a).

In soils, surface and ground waters arsenic is found in –3, 0, +3 and +5 oxidation states, but its prevalent forms are the inorganic species, arsenate [As(V)] and arsenite [As(III)]. Relative to other oxyanion-forming elements, arsenic is among the most problematic in the environment because of its relative mobility over a wide range of redox conditions. Arsenic is relatively mobile under reduced conditions (Smedley and Kinniburgh 2002). It may occur as methylated forms in environmental systems, but these organic species are usually rare in soils and surface waters. The methylated monomeric arsenic species are: monomethylarsonic acid ($H_2AsO_3(CH_3)$, $MMAs^{v}$), methylarsinous acid (H_2AsO_2-CH_3,$MMAs^{III}$), dimethylarsinic acid ($HAsO_2$-$(CH_3)_2$, $DMAs^{v}$), dimethylarsinous acid (HAsO-$(CH_3)_2$, $DMAs^{III}$), trimethylarsine oxide (AsO-$(CH_3)_3$, $TMAsO^{v}$), and trimethylarsine (As-$(CH_3)_3$, $TMAs^{III}$). Methylation can be carried out by a variety of organisms ranging from bacteria to fungi to mammals and is believed to be part of a detoxification mechanism in living organisms. The arsenic compounds present in natural enviroments have recently been comprehensively reviewed by Francesconi and Kuehnelt (2002).

The mobility of arsenic compounds in soils is affected by sorption/desorption on/from soil components or co-precipitation with metal ions. The importance of oxides (mainly Fe-oxides) in controlling the mobility and concentration of arsenic in natural environments has been studied for a long time (Livesey and Huang 1981; Frankenberger 2002 and references there in; Smedley and Kinniburgh 2002). Because the elements which correlate best with arsenic in soils and sediments are iron, aluminum and manganese, the use of Fe salts (as well as Al and Mn salts) is a common practice in water treatment for the removal of arsenic. The coprecipitation of arsenic with ferric or aluminum hydroxide has been a practical and effective technique to remove this toxic element from polluted waters

(Scott et al. 1995; Rancourt et al. 2001; Thirunavukkarasu et al. 2003). Iron-arsenic coprecipitates have been found in natural environments (Pichler et al. 1999; Frankenberger 2002).

Arsenite is 25–60 times more toxic than arsenate, which mainly arise from its state as H_3AsO_3 at pH < 9.0 as compared to the charged arsenate species which predominate in a wide pH range ($H_2AsO_4^-$ between 2.5 and 7, $HAsO_4^{2-}$ between pH 7 and 12) (Frankenberger 2002; Smedley and Kinniburgh 2002). Bioavailability of arsenic in soil may be affected by inorganic (mainly phosphate added as fertilizer) or naturally occurring organic molecules, which may affect the sorption/desorption processes of this metalloid onto/from soil components (Violante et al. 2005a,b). From the toxicological point of view ingestion of inorganic arsenic can result in both cancer (skin, lung and urinary bladder) and non cancer effects (skin lesions). Recent data suggests that some methyl arsenic species ($MMAs^{III}$ and $DMAs^{III}$) can be as toxic or more toxic than inorganic species (arsenate and arsenite) (Francesconi and Kuehnelt 2002; Le 2002).

The aim of this Chapter is to provide the current state of knowledge on the factors (pH, surface coverage, residence time, presence of organic and inorganic ligands) which influence the sorption/desorption processes of arsenic by soil minerals and soils integrating the existing literature on this subject with our recent findings.

2 Sorption onto Soil Components

Arsenate and arsenite are sorbed primarily by chemisorption at reactive sites of variable charge minerals, such as metal oxides and short-range ordered aluminosilicates (allophane, imogolite) and at the edges of phyllosilicates (Manceau 1995; Raven et al. 1998; Frankenberger 2002; Violante and Pigna 2002; Ona-Nguema et al. 2005). The carbonates also play an important role in the arsenate sorption of calcareous soils in the pH range 9–12 (Goldberg and Glaubig 1988).

Usually, elements in anionic form are not easily sorbed on soil organic matter, but arsenate and arsenite were found to be bound to natural organic matter probably held to organic groups through a bridging hydrolytic species of Al and Fe (Thanabalasingan and Pickering 1986; McBride 2000). Binding mechanisms of arsenite and arsenate to dissolved humic acids have been proposed (Buschmann et al. 2006). At all pH values, arsenate was more strongly bound than arsenite maximum binding being around pH 7.0 (Buschmann et al. 2006; Ritter et al. 2006).

Sorption of arsenic onto minerals and soils varies with pH. Frost and Griffin (1977) reported that arsenate sorption by kaolinite and montmorillonite exhibited a maximum at pH 4.0–6.0, whereas arsenite was sorbed steadily from pH 4.0 to 9.0 on kaolinite and peaked at pH 7.0 on montmorillonite. Later, Manning and Goldberg (1996a) found that distinct arsenate adsorption maxima occurred at approximately pH 5.0 for kaolinite, 6.0 for montmorillonite and 6.5 for illite. Sorption of arsenite onto phyllosilicate clay minerals has also been studied (Manning and Goldberg 1997a).

Arsenate and arsenite have different trend in sorption on variable charge minerals (Inskeep et al. 2002). Many studies have demonstrated that arsenite is sorbed on Al-oxides, phyllosilicates and calcite in a lesser extent than arsenate, whereas the opposite is true for iron oxides (Frankenberger 2002; Martin et al. 2007a). The sorption of arsenate onto Fe- and Al-oxides usually increases by decreasing pH, but rapidly decreases above pH 7.0, probably because at pH > 7.0 the surfaces of these oxides are negatively charged, their point of zero charge (pzc) being approximately 6.5–7.5 (Hsu 1989; Cornell and Schwertmann 1996; Kampf et al. 2000). The sorption of arsenite onto Fe oxides is highly pH dependent with the envelope centered at about pH 9.0. Raven et al. (1998) showed that arsenite has a greater sorption capacity on ferrihydrite and goethite than arsenate except at very low solution concentrations. Furthermore, Martin et al. (2007b) ascertained that iron oxides can sorb more arsenite than arsenate, although the K of Langmuir isotherms which is a constant related to the binding energy is always lower for arsenite than for arsenate.

Chemical behavior of arsenate is similar to that of phosphate and may form different surface complexes (inner-sphere complexes) on inorganic soil components: monodentate, bidentate-binuclear and bidentate-mononuclear complex in different proportions depending on pH and surface coverage (Hsia et al. 1994; Sun and Doner 1996; Fendorf et al. 1997; O'Reilly et al. 2001). Arsenite and arsenate seem to form similar surface complexes with metal oxides with arsenate more strongly held on these soil components. Surface complexes of arsenate and arsenite on iron oxides have been studied using infrared (Sun and Doner 1996) and extended x-ray absorption fine structure (EXAFS) spectroscopy (Manceau 1995; Waychunas et al. 1996; Fendorf et al. 1997; Farquhar et al. 2002; Ona-Nguema et al. 2005). The general consensus is that both arsenate and arsenite form mainly bidentate binuclear complexes with two adjacent iron octahedral corner sites with a slight longer d(As-Fe) for arsenite. However, arsenite seems to form both inner- and outer-sphere complexes onto Fe-oxides and outer-sphere complexes on Al oxides (Goldberg and Johnston 2001; Arai et al. 2001). Recently EXAFS study (Ona-Nguema et al. 2005) indicates that at high surface coverage arsenite forms bidentate mononuclear edge-sharing and

bidentate binuclear corner-sharing onto ferrihydrite and hematite, but dominantly bidentate binuclear corner-sharing sorption complexes onto goethite and lepidocrocite (with minor amount of monodentate mononuclear edge). Unfortunately, no information is available on the possible complexes of methylated forms of arsenic onto soil components.

Goldberg (2002) found that arsenate and arsenite sorption on amorphous Fe-oxide and Al-oxide as a function of solution pH showed negligible ionic strength dependence in the range 0.02 to 0.1 mol L^{-1}. However, arsenite showed decreasing sorption with increasing ionic strength in the range 0.1 to 1.0 mol L^{-1}, indicative of outer-sphere sorption mechanism. Arsenate and arsenite sorption lowers the pzc of Fe-oxides, but some authors showed that the pzc of Al-oxides shifts to lower pH values with increasing arsenate concentration, but does not shift to lower pH in the presence of arsenite (Jain et al. 1999; Goldberg and Johnston 2001; Martin et al. 2007b).

Sorption of arsenite on the surfaces of Mn-oxide facilitate the oxidation of arsenite to arsenate (Oscarson et al. 1981). In some environments contaminated with arsenite, the presence of Mn oxides decreases the potential toxicity of arsenite by converting arsenite to the less toxic arsenate and the subsequent sorption of this species (Smith et al. 1998). More recently, Tournassat et al. (2002) studied the oxidation of arsenate in 0.011 mol L^{-1} arsenite suspension of well crystallized hexagonal birnessite and found that the surficial reaction sites are likely located on the edges of H-birnessite layers rather than on the basal planes. A protonated manganese precipitate (probably krautite) formed after 74 hrs of reaction whose long fibers were aggregated at the surfaces of birnessite. This study demonstrated that the oxidation reaction As(III)-MnO_2 transforms the toxic arsenite to a less toxic aqueous arsenate species, which subsequently precipitates with Mn^{2+} as a mixed As-Mn solid characterized by a low solubility product. Partial oxidation of arsenite on the surfaces of goethite has been demonstrated by Sun and Doner (1998) who found that after 20 days, more than 20% of arsenite, sorbed on the surfaces of goethite, was oxidized to arsenate. Manning and Goldberg (1997a) demonstrated that oxidation of arsenite to arsenate was enhanced in the presence of phyllosilicates by heterogenous reactions with components on the surfaces of clay minerals. The relationship between soil properties and sorption of arsenite and arsenate has also been studied (Manning and Goldberg 1997b).

Sorption of methyl arsenic onto metal oxides has received scant attention. Lafferty and Loeppert (2005) found that MMAs(III) and DMAs(III) were not appreciably sorbed onto goethite or ferrihydrite within the pH range of 3 to 11, while arsenite was strongly sorbed to both the oxides. In contrast, MMAs(V) and arsenate were sorbed from pH 3 to 10 in great

amounts on the iron oxides, whereas DMAs(V) was sorbed only at pH below 7 on goethite and below 8 on ferrihydrite. These authors demonstrated that DMAs(V) is specifically sorbed by iron oxides only at pH values lower than the pzc, with no sorption at pH values above pzc. The retention behaviour of arsenate and MMAs(V) were similar, but with a weaker bond between MMAs(V) and iron-oxide surfaces than between arsenate and iron oxides (especially at high pH values). According to these authors the difference in apparent bonding strength of MMAs(V) and arsenate might be due to the electron donating characteristics of the methyl group of MMAs(V).

3 Influence of Competing Anion

The presence of inorganic and organic ligands affects the sorption of arsenic onto soil minerals and soils by competing for available binding sites and/or reducing the surface charge of the sorbents (Barrow 1992; Manning and Goldberg 1996a,b; Frankenberger 2002; Violante and Pigna 2002; Violante et al. 2005a,b; 2008, and references there in). The competition in sorption is affected by the affinity of the competing anions for the surfaces of the sorbents, the nature and surface properties of the minerals and soils, the surface coverage and the reaction time.

Goldberg (2002) found no evidence of any competition in sorption of arsenate and arsenite on Al or Fe-oxides and montmorillonite, but only a small and apparent competitive effect of equimolar arsenate on arsenite sorption on kaolinite and illite. The minor competitive effect in this study was due to the small concentrations of arsenic which is very low for saturation site. Competition for sorption sites is evident by increasing the surface coverage of the sorbents. Arsenate prevents arsenite sorption on metal oxides when the surfaces of the sorbents are saturated by the anions (Jain and Loeppert 2000; Violante and Pigna 2002).

The effect of phosphate on the sorption/desorption of arsenic in soil environments has received great attention, since application of phosphate fertilizers is a management practice that can have a direct effect on the concentration of arsenic in soil solution and may enhance arsenic's mobility (Manning and Goldberg 1996b; Smith et al. 1998; Jain and Loeppert 2000; Hongshao and Stanforth 2001; Frankenberger 2002 and references there in; Violante and Pigna 2002). Violante and Pigna (2002) studied the competition in sorption between phosphate and arsenate on selected phyllosilicates, metal oxides, and soil samples. They found that Mn, Fe and Ti oxides and phyllosilicates particularly rich in Fe (nontronite, ferruginous

smectites) were more effective in sorbing arsenate than phosphate after 24 hrs of reaction, but more phosphate than arsenate was sorbed on noncrystalline Al precipitation products, gibbsite, boehmite, allophane, and kaolinite. These authors found that competitiveness between the anions also changed at different pH values. In particular phosphate inhibited arsenate sorption more in neutral and alkaline systems than in acidic systems.

Smith et al. (2002) studied the effect of phosphate on the sorption of arsenate and arsenite by an Oxisol, a Vertisol and two Alfisols. The presence of phosphate (0.16 mmol L^{-1}) greatly decreased arsenate sorption by soil containing low amounts of Fe oxides (< 100 mmol kg^{-1}: Alfisols. Fig. 1A), indicating competitive sorption between phosphate and arsenate for sorption sites. In contrast, the presence of a similar amount of phosphate had relatively little effect on the amount of arsenate sorbed by soils (Oxisol) with high iron content (> 800 mmol kg^{-1}: Fig. 1B). A similar effect of phosphate on arsenite sorption was observed in low sorbing Alfisols (Fig. 2A) and high affinity Oxisol (Fig. 2B). However, the amount of arsenite sorbed by the Oxisol was much greater than the Alfisol.

Because the final surface coverage of competitive ligands onto the surfaces of the sorbents seems to have a great effect on their sorption, it appeared interesting to carry out experiments on the competitive sorption of phosphate and arsenate at pH 5.0 onto ferrihydrite or a noncrystalline Al-oxide [$Al(OH)_x$] at a surface coverage of each ligand of 50 or 100% and after 5–720 hrs of reaction (Del Gaudio, 2005). The surface area of ferrihydrite and $Al(OH)_x$, determinated by the method of Quirk (1955), was respectively of 230 and 135 m^2 g^{-1}. The initial arsenate added/phosphate added molar ratio (ri) was 1, but some experiments were carried out at ri of 0.5. The anions were added to the sorbents as a mixture (*AsO_4+PO_4* [ri = 1] and *AsO_4+2PO_4* [ri = 0.5]) or by adding arsenate 24 hrs before phosphate (*AsO_4 before PO_4*) or adding phosphate 24 hrs before arsenate (*PO_4 before AsO_4*).

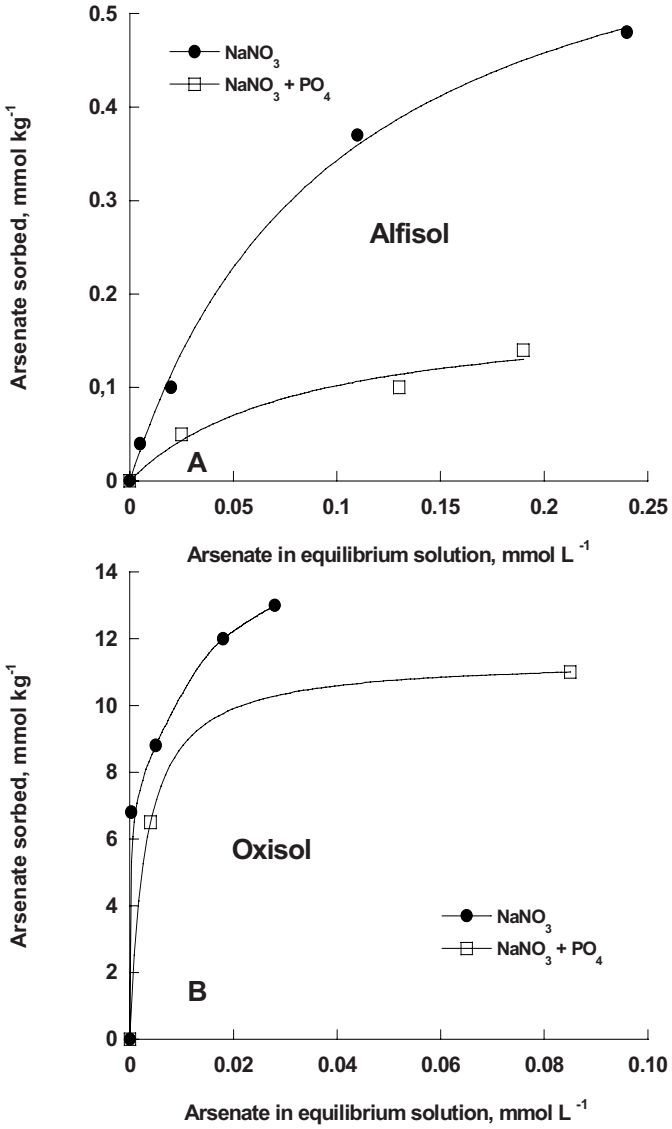

Fig. 1. Arsenate sorption (mmol kg^{-1}) on Alfisol (**A**) and on Oxisol (**B**) in the presence of sodium nitrate or sodium nitrate + phosphate (PO_4). Redrawn from Smith et al. (2002).

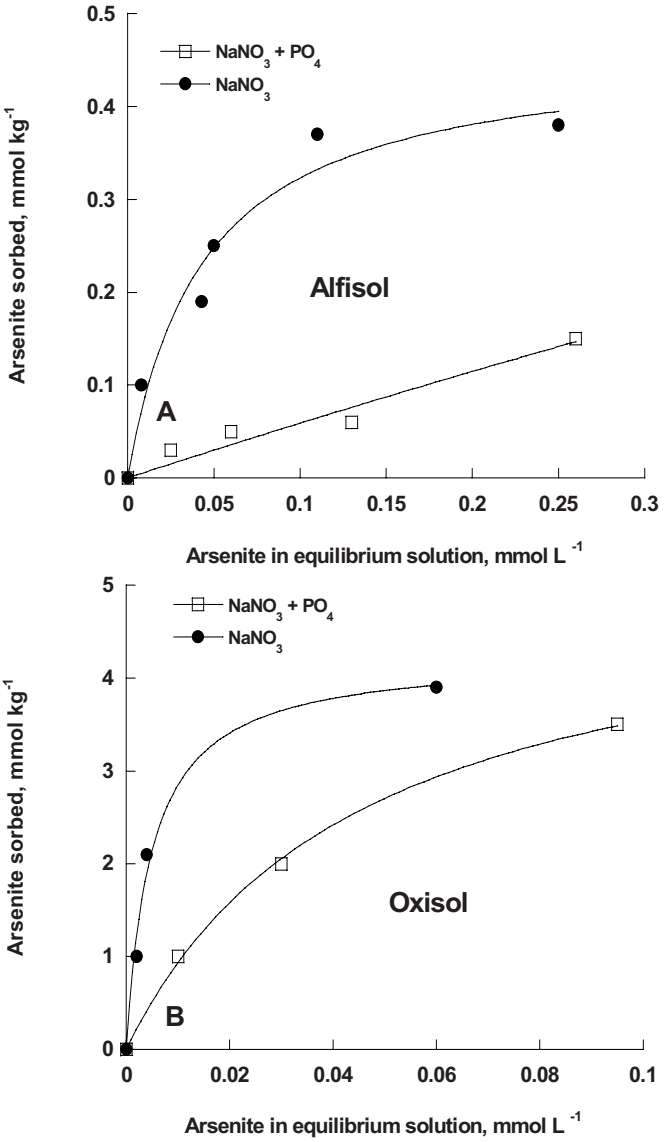

Fig. 2. Arsenite sorption (mmol kg^{-1}) on Alfisol (**A**) and on Oxisol (**B**) in the presence of sodium nitrate or sodium nitrate + phosphate (PO_4). Redrawn from Smith et al. (2002).

Figures 3 and 4 show the amounts of arsenate sorbed onto ferrihydrite and Al(OH)x after 24 hrs of reaction with a surface coverage of arsenate and phosphate respectively of 50 or 100%. The numbers in parenthesis indicate the efficiency (in percentage) of phosphate in preventing sorption of arsenate calculated according to the expression of Deb and Datta (1967).

Efficiency of P (%) = {1–[As sorbed in the presence of P/As sorbed when applied alone]}×100.

It appears evident that phosphate prevented arsenate sorption more onto Al(OH)x than ferrihydrite and more when the surface coverage of both the ligands onto the sorbents was near 100% in comparison to that at 50%. In fact, in the AsO_4+PO_4 systems the efficiency of phosphate in inhibiting arsenate sorption onto Al(OH)x was of 49% and 79% respectively at 50% and 100% of surface coverage, whereas on ferrihydrite it was much lower viz., 8% and 41%, respectively. The sequence of anion addition strongly influenced arsenate sorption. Lower amounts of arsenate were sorbed in *PO_4 before AsO_4* system and greater quantities of arsenate were sorbed in *AsO_4 before PO_4* system as referred to AsO_4+PO_4 system (Figs. 3 and 4).

The effect of other inorganic anions (sulfate, molybdate, silicate), low molecular mass organic ligands (LMMOLs, such as oxalate, malate, citrate, tartrate and succinate), and fulvic or humic acid on the sorption of arsenate and arsenite onto variable charge minerals and soils has been studied (Roy et al. 1986; Grafe et al. 2001; Liu et al. 2001; Violante et al. 2005a,b).

Sulfate poorly prevents arsenate sorption onto metal oxides and soils (Wu et al. 2001; Inskeep et al. 2002; Violante et al. 2005b). Violante et al. (2005b) found that high concentrations of sulfate (sulfate/arsenate molar ratio (rf) 4–10) retarded but not prevented arsenate sorption onto ferrihydrite (see their Fig. 15.10) or other metal oxides. Roy et al. (1986) showed that the sorption of arsenate by two soils (an Ultisol and a Typic Apludults) was reduced in the presence of molybdate.

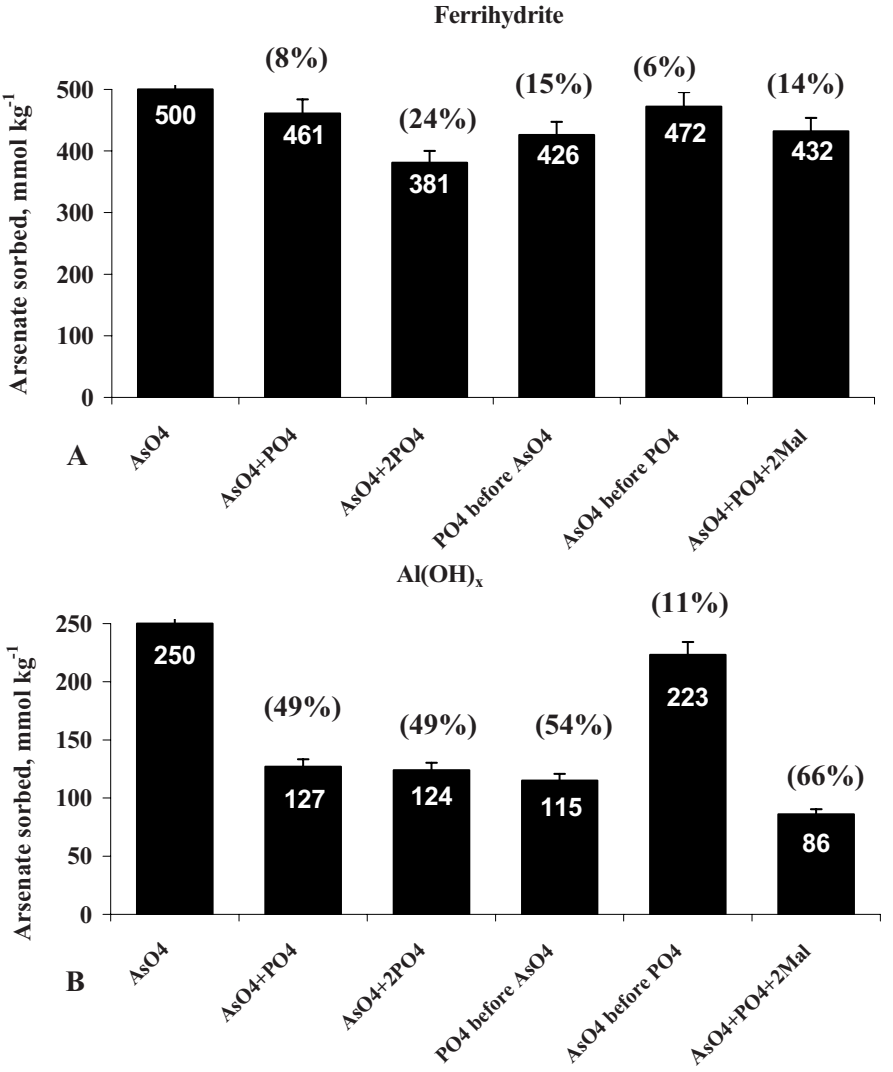

Fig. 3. Sorption of arsenate (AsO_4) onto ferrihydrite or $Al(OH)_x$ in the presence of phosphate (PO_4) or phosphate and malate (Mal) at 50% surface coverage of arsenate and at initial AsO_4/PO_4 molar ratio of 1.0 or 0.5. Arsenate and phosphate were added as a mixture (*AsO_4+PO_4*: *AsO_4+2PO_4*) or phosphate was added 24 hrs before arsenate (*PO_4 before AsO_4*) or arsenate was added 24 hrs before phosphate (*AsO_4 before PO_4*). Arsenate, phosphate and malate were added as a mixture (AsO_4+ PO_4/Mal molar ratio of 1). The numbers in parenthesis indicate the effectiveness of phosphate in preventing arsenate sorption. From Del Gaudio (2005).

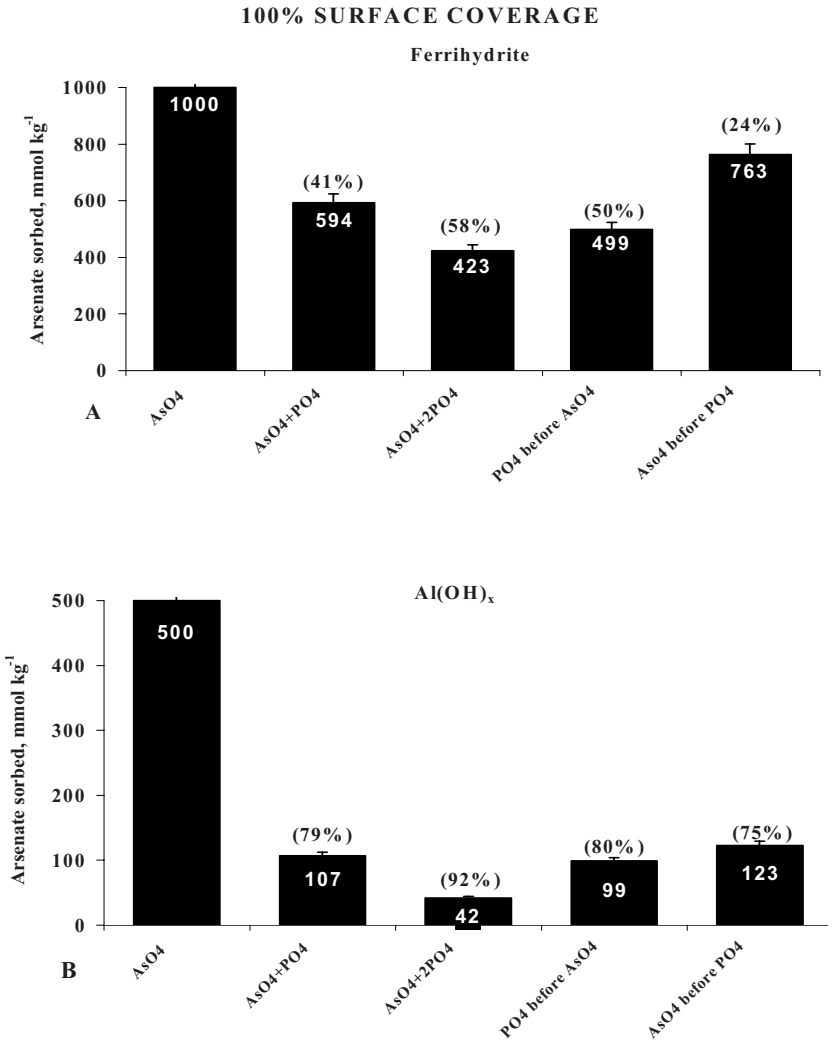

Fig. 4. Sorption of arsenate (AsO_4) onto ferrihydrite or $Al(OH)_x$ in the presence of phosphate (PO_4) or phosphate and malate (Mal) at 100% surface coverage of arsenate and at initial AsO_4/PO_4 molar ratio of 1.0 or 0.5. Arsenate and phosphate were added as a mixture (*AsO_4+PO_4*: *AsO_4+2PO_4*) or phosphate was added 24 hrs before arsenate (*PO_4 before AsO_4*) or arsenate was added 24 hrs before phosphate (*AsO_4 before PO_4*). The numbers in parenthesis indicate the effectiveness of phosphate in preventing arsenate sorption. From Del Gaudio (2005).

The kinetics of sorption of arsenite and arsenate in the presence of sorbed silicic acid have been only recently examined (Waltham and Eick 2002). These authors demonstrated that the sorption of silicic acid (added 60 h before arsenic) decreased the rate and the total amount of arsenic sorbed. The amount of arsenite sorbed decreased as the surface concentration of silicic acid increased. Furthermore, the inhibition of arsenite sorbed ranged from about 4% at a pH of 6 and 0.1 mM silicic acid up to 40% at a pH of 8 and 1 mol L^{-1} silicic acid. In contrast, silicic acid reduced the rate of arsenate sorption which decreased by increasing pH and silicic acid concentration, but the total quantity of arsenate sorbed remained nearly constant, indicating that arsenate was able to replace silicate.

Grafe et al. (2001) found that arsenate sorption onto goethite was reduced by humic and fulvic acid, but not by citric acid, whereas arsenite sorption was decreased by all three organic acids between pH 3.0 and 8.0 in the order of citric acid > fulvic acid > humic acid. Del Gaudio (2005) showed that the inhibition of malate (Mal) on arsenate sorption was negligible onto ferrihydrite (100% Arsenate surface coverage) even when malate was added before arsenate but not onto Al(OH)x. At an initial Mal/As molar ratio of 1, the sorption of arsenate onto Al(OH)x after 24 hrs of reaction was reduced by 40% (Fig. 5).

4 Sorption in Ternary Systems

Sorption of arsenate or arsenite in ternary systems has received scant attention. Some experiments on the sorption of arsenate onto ferrihydrite or Al(OH)x in the presence of phosphate and malate (50% surface coverage of arsenate initial PO_4/AsO_4 molar ratio of 1 and Mal/PO_4+AsO_4 of 1; *AsO_4+PO_4+2Mal* systems) were carried out by Del Gaudio (2005). In *AsO_4+PO_4+2Mal* systems arsenate sorption was reduced much more on Al(OH)x (66%) than on ferrihydrite (14%) (Fig. 3). Furthermore, it was found that the rf values were greater in *AsO_4+PO_4* system than in *AsO_4+PO_4+2Mal* system (0.5 vs 0.4), whereas, for ferrihydrite as the sorbent, the opposite was true (1.10 vs 1.05; data not shown). These findings demonstrate that malate competed with arsenate more for the surface sites of Al(OH)x, than for those of ferrihydrite, whereas the opposite was true for phosphate. The sorption of three or more ligands onto soil components deserves attention.

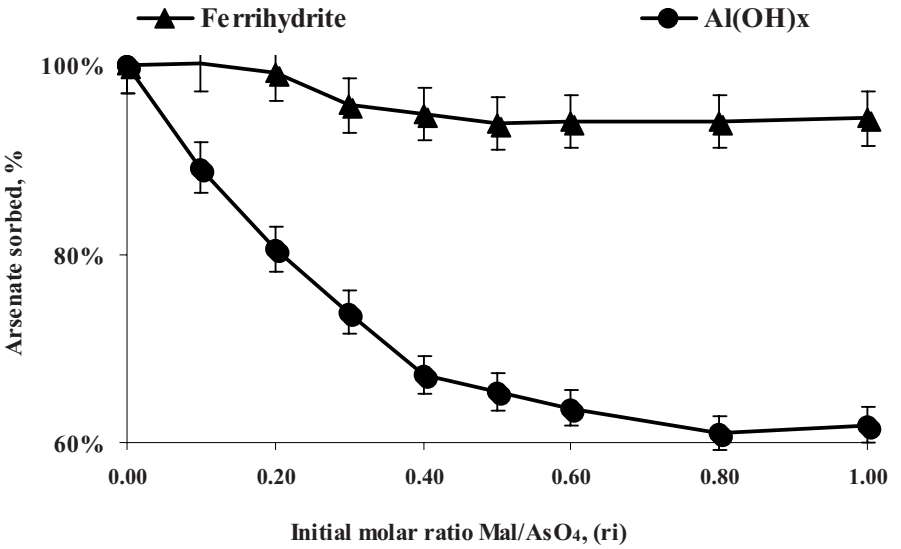

Fig. 5. Influence of increasing concentrations of malate (Mal) on the sorption of arsenate (*AsO_4*; 100% surface coverage) onto ferrihydrite or $Al(OH)_x$ at pH 5.0. Malate was added 24 hrs before arsenate (*Mal before AsO_4*). From Del Gaudio (2005).

5 Kinetics Sorption of Arsenate

The amounts of arsenate and arsenite sorbed onto soil components are affected by the time of reaction and presence of foreign ligands (Raven et al. 1998; Grafe et al. 2001; Frankenberger 2002; Violante and Pigna 2002; Pigna et al. 2006). We have carried out experiments on the kinetics of sorption of arsenate onto ferrihydrite and Al(OH)x in the absence or presence of phosphate and both phosphate and malate. Table 1 shows the amounts of arsenate and phosphate sorbed onto Al(OH)x at pH 5.0 after 0.03–168 hrs when these anions were added alone or as a mixture at 50% or 100% of surface coverage, whereas Fig. 6 shows the sorption of arsenate onto ferrihydrite and $Al(OH)_x$ (50% surface coverage) in the absence or presence of phosphate (*AsO_4+PO_4*) or phosphate and malate (*AsO_4+PO_4+2Mal*) during the first 24 hrs of reaction (Del Gaudio 2005; Violante and Pigna 2007 unpublished data). It appears evident that each ligand inhibited the sorption of the other; in fact, at 50% of surface coverage

(Table 1) arsenate and phosphate were completely sorbed onto $Al(OH)_x$ within 3–5 hrs of reaction when added alone, but only after more than 168 hrs when added as a mixture. When the oxyanions were added together the arsenate sorbed/phosphate sorbed molar ratio (rf) continuously increased with time from 0.21 after 0.03 hrs to 0.94 after 168 hrs. The rf values were initially < 1, since the sorption of phosphate was faster than that of arsenate. The rf reached the value of 1 only after 720 hrs (data not shown), indicating that all the ligands added were fixed on the surfaces of the oxide. On ferrihydrite at 50% surface coverage the rf values were initially greater than 1 and then decreased with time up to 1 (data not shown). Similar results were obtained by using hematites of different morphology and surface properties (Pigna et al. 2003). Clearly, an initial faster sorption of an anion onto the surface of a given sorbent affected the sorption of the other. A reduction in surface charge because of the initial sorption of phosphate or arsenate may also differently reduce the rate of anions sorption, which may be responsible for the observed residence time effect.

When the surface coverage of each ligand was 100%, the rf values increased more slowly from 0.19 to 0.41, as the time increased from 0.03 hrs to 168 hrs (Table 1). In fact, rf values increased 4.4 times from 0.03 to 48 hrs when the surface coverage was 50% and 2 times when the surface coverage was 100%. These findings indicate that when the surface coverage was high, being the sites not available for all the ligands added, there was a strong competition for sorption sites between arsenate and phosphate anions. Even after a reaction time of 700 to 1000 hrs the rf values were < 0.6 (data not shown). In the presence of both phosphate and malate (*AsO_4+PO_4+2Mal*) arsenate sorption was strongly prevented; after 360 hrs of reaction 71% and 24% of arsenate was sorbed onto ferrihydrite and $Al(OH)_x$ respectively (data not shown). From the results described before (Table 1; Fig. 6) it can be concluded that both competition for sorption sites and change in the surface charge of the sorbents occur simultaneously to explain the competition in adsorption between ions.

The kinetics sorption data of arsenate onto ferrihydrite and Al(OX)x were tested by different models (first order, parabolic diffusion, and Elovich). The fit for the sorption data was obtained best using Elovich model (Fig. 7). Similar results were obtained by Pigna et al. (2006). At 50% surface coverage, the kinetics of sorption of arsenate on ferrihydrite could be explained best by assuming two processes, the first one (fast sorption) operating during the first 0.167 hrs of reaction when arsenate was added alone or during the first 24 hrs in the presence of phosphate (*AsO_4+PO_4* system) or phosphate and malate (*AsO_4+PO_4+2Mal* system) (Fig. 7A). A similar trend was not obtained using $Al(OH)_x$ as sorbent

(Fig. 7B). This behaviour may be attributed to the higher affinity of arsenate for the iron than for aluminium oxides.

Table 1. Kinetics of reaction of arsenate (AsO_4) and phosphate (PO_4) onto $Al(OH)_x$ when added alone or as a mixture (AsO_4+ or PO_4+) (initial AsO_4/PO_4 molar ratio of 1) at 50% or 100% surface coverage. rf indicates the AsO_4 sorbed/PO_4 sorbed molar ratio (authors' unpublished data, 2007)

Time (hrs)	AsO_4 sorbed (mmol Kg^{-1})	PO_4 sorbed (mmol Kg^{-1})	AsO_4+ sorbed (mmol Kg^{-1})	PO_4+ sorbed (mmol Kg^{-1})	rf AsO_4/PO_4
50% Surface coverage*					
0.03	143	162	32	154	0.21
0.5	166	196	60	157	0.38
1	186	219	81	198	0.41
3	234	236	111	225	0.49
24	250	245	198	244	0.81
48	250	249	224	241	0.92
168	250	233	232	247	0.94
100% Surface coverage**					
0.03	155	193	27	139	0.19
0.5	167	263	21	176	0.12
1	216	299	49	193	0.25
3	260	345	57	252	0.22
24	407	491	95	382	0.25
48	486	495	189	466	0.40
168	498	496	197	476	0.41

*Two hundred fifty mmol AsO_4 added per kg of sorbent.
**Five hundred mmol added AsO_4 per kg of sorbent.

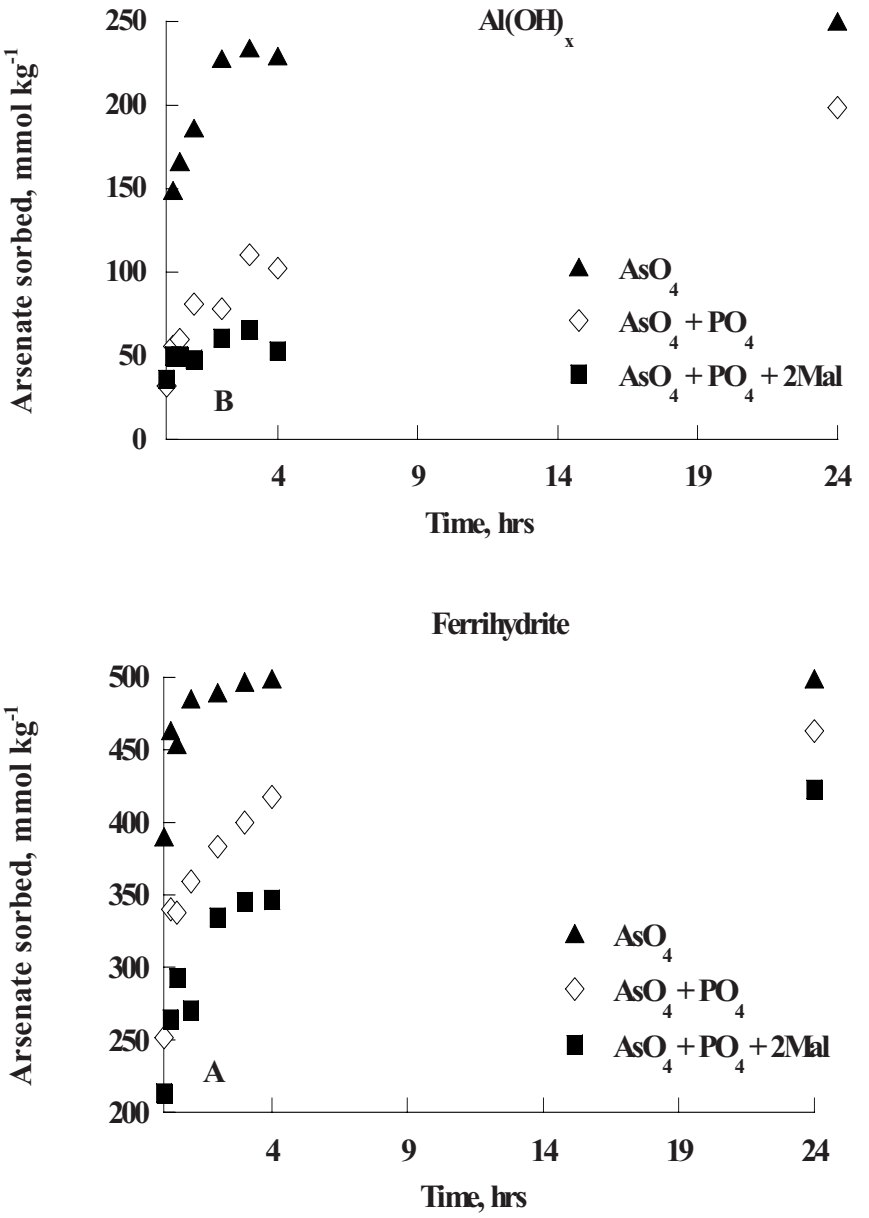

Fig. 6. Kinetics of arsenate (*AsO_4*) sorption onto ferrihydrite (A) or $Al(OH)_x$ (B) at pH 5.0 in the absence or presence of phosphate (*PO_4*) or phosphate and malate (Mal). Initial PO_4/AsO_4 molar ratio of 1 (*AsO_4 + PO_4*) and *AsO_4 + PO_4*/Mal molar ratio of 1 (*AsO_4+PO_4 +2Mal*). Arsenate was added at 50% of surface coverage (authors' unpublished data, 2007).

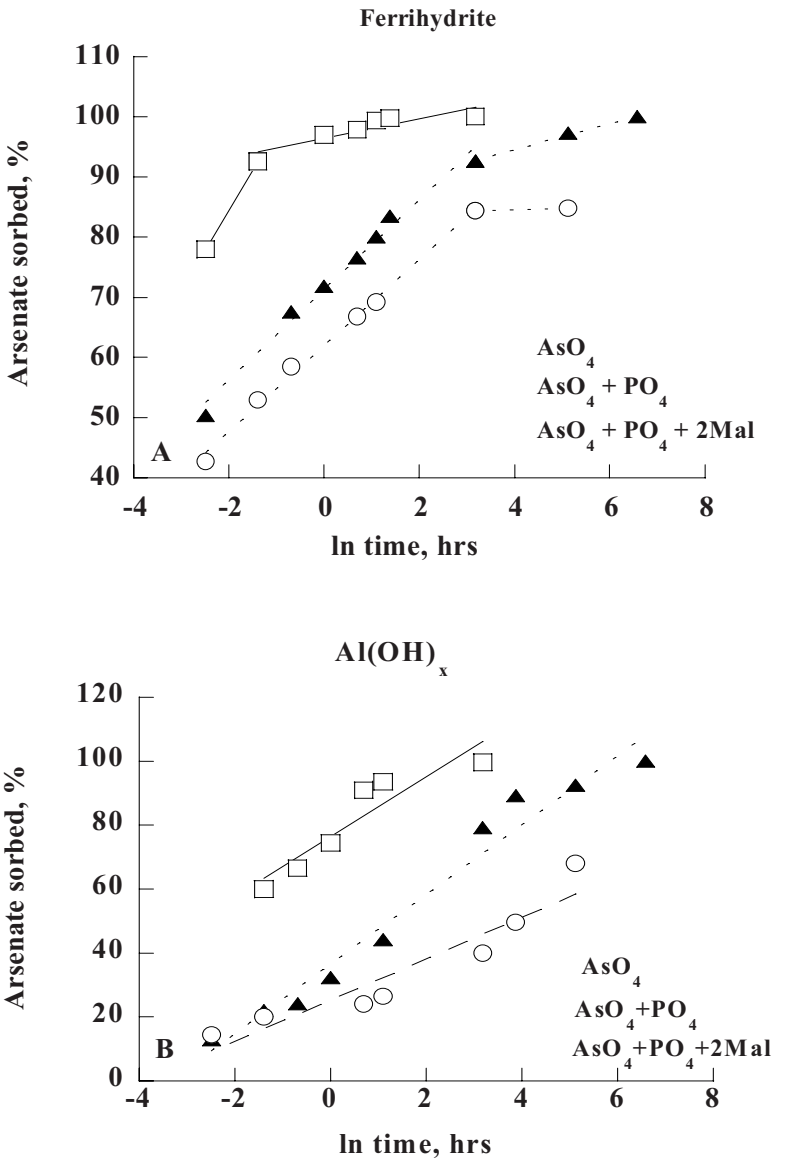

Fig. 7. Kinetics of arsenate (*AsO_4*) sorption onto ferrihydrite (A) or $Al(OH)_x$ (B) at pH 5.0 in the absence or presence of phosphate (*PO_4*) or phosphate and malate (Mal). Initial PO_4/AsO_4 molar ratio of 1 (*AsO_4 + PO_4*) and *AsO_4 + PO_4*/Mal molar ratio of 1 (*AsO_4+PO_4 +2Mal*). Arsenate was added at 50% of surface coverage. The fit for the sorption data was obtained best using Elovich model (authors' unpublished data, 2007).

6 Desorption of Arsenate

Desorption of arsenic by foreign ligands (mainly phosphate) has received attention particularly in the last years. Goh and Lym (2005) evaluated the extractability of arsenate from the fine fraction of an acidic soil deliberately contaminated with arsenate and aged for more than 220 days by various salts such as Na_3PO_4, Na_2CO_3, Na_2SO_4 and NaCl. The results of arsenic extraction as a function of reaction time in the presence of phosphate, sulfate, carbonate and chloride (0.005 M) are reported in Fig. 8. Both chloride and sulfate solutions extracted less than 20% of arsenic from the soil. The percentages of arsenate extracted by carbonate were slightly higher than those mobilized by chloride or sulfate. Phosphate demonstrated the highest arsenic extraction efficiency among the anions used. The percentage of arsenic extracted by phosphate increased rather rapidly within short reaction times, and they continued to increase gradually toward equilibration (Fig. 8). Therefore, the effectiveness of the anions in mobilizing arsenic from the soil followed the order: $PO_4 >> CO_3 > SO_4 \approx Cl$.

O'Reilly et al. (2001) studied the effect of sorption residence time on arsenate desorption by phosphate (phosphate/arsenate molar ratio of 3) from goethite at different pH values. Initially, desorption was very fast (35% arsenate desorbed at pH 6.0 within 24 hrs) and then slowed down. Total desorption increased with time reaching about 65% total desorption after 5 months. These authors found no measurable effect of aging on desorption of arsenate in the presence of phosphate. Furthermore, desorption results at pH 4.0 were similar to the desorption behaviour at pH 6.0. On the contrary, Arai and Sparks (2002) demonstrated that the longer the residence time (3 days–1 year), the greater was the decrease in arsenate desorption by phosphate from a bayerite.

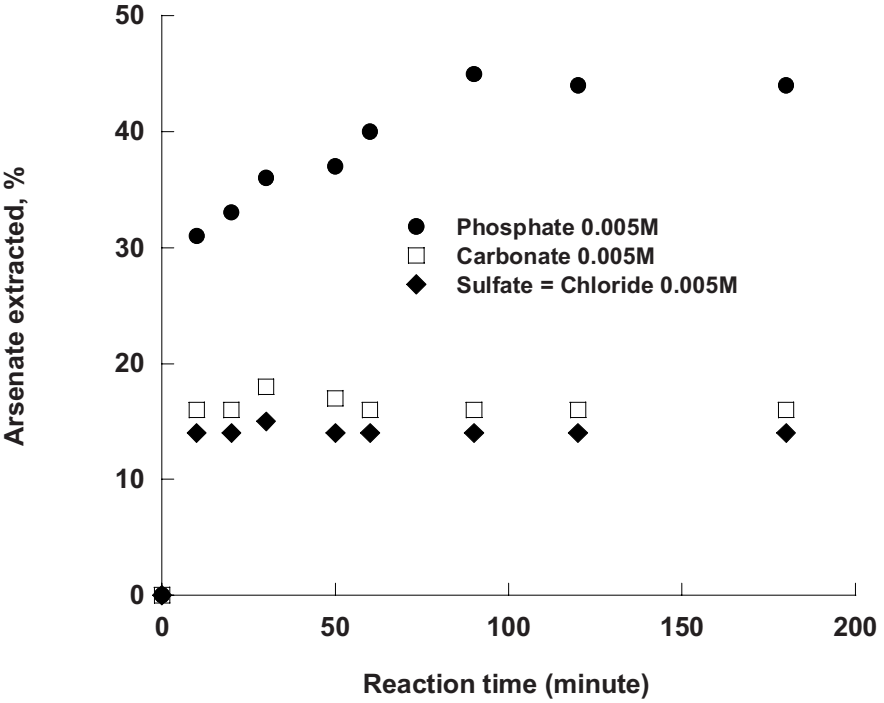

Fig. 8. Arsenic extraction from a reddish brown tropical soil deliberately contaminated with arsenate as a function of reaction time by 0.005M chloride, sulphate, carbonate and phosphate. Redrawn from Goh and Lym (2005).

The desorption of arsenate previously sorbed onto Fe- or Al-oxides or onto an Andisol containing 42% of allophanic materials (Vacca et al. 2002) by phosphate has been demonstrated to be affected by time of reaction, residence time of arsenate onto the surfaces and the pH of the system (Pigna et al. 2006; Pigna et al. 2007, unpublished data). Figure 9 shows the desorption of arsenate at pH 6.0 (phosphate/arsenate molar ratio of 4) when phosphate was added onto the soil (Andisol) sample 1, 5 or 15 days after arsenate (surface coverage of arsenate about 60%). After 60 days of reaction, 55% of arsenate was desorbed by phosphate when the residence time of arsenate onto the surfaces of the Andisol was 1 day, but 35 and 20% of arsenate was desorbed by phosphate with increase in the residence time up to 5 and 15 days. Further, it was also observed that by keeping the

surface coverage and residence time constant the desorption of arsenate by phosphate increased by increasing the pH of the system. The arsenate desorbed after 24 hrs of reaction ranged from 41% at pH 4.0 to 73% at pH 8.0 (data not shown). Pigna et al. (2006) have reported that the desorption of arsenate by phosphate from iron and aluminum oxides was affected by the crystallinity of the sorbents.

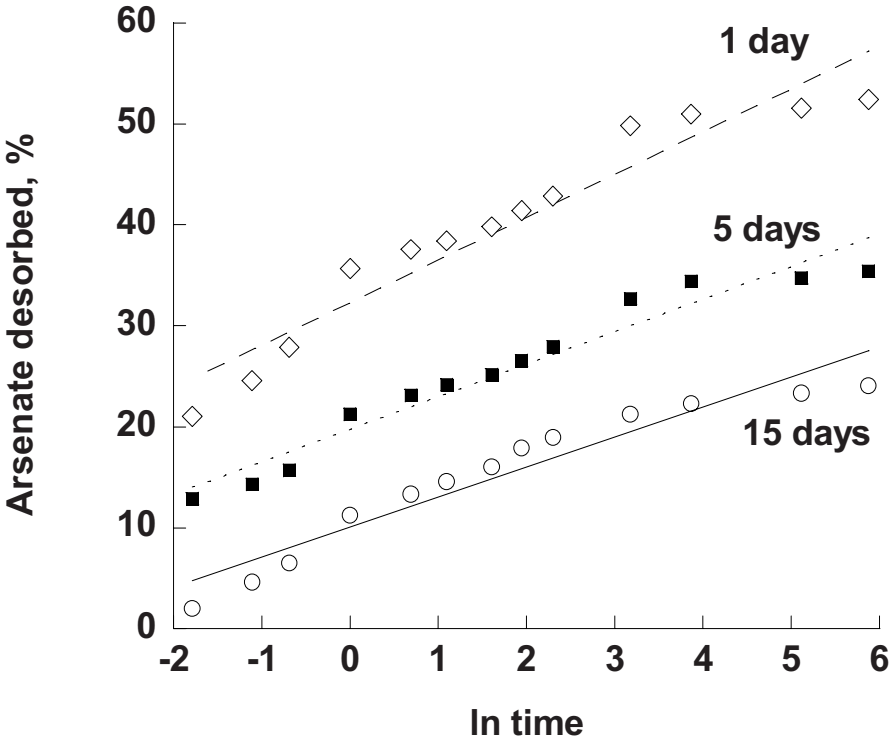

Fig. 9. Desorption of arsenate (AsO_4) from Andisol at pH 6.0 (phosphate/arsenate molar ratio of 4) when phosphate (PO_4) was added 1, 5 or 15 days after arsenate. Surface coverage of arsenate was about 60% (authors' unpublished data, 2007).

Desorption of arsenate, MMAs(V) and DMAs(V) from goethite and ferrihydrite by phosphate and sulfate was studied by Lafferty and Loeppert (2005). These arsenic compounds were desorbed more efficiently by phosphate than sulfate. In desorption envelopes, the amount of arsenate desorbed generally increased as the number of methyl groups increased

[arsenate < MMAs(V) < DMAs(V)]. Desorption of MMAs(V) by phosphate from ferrihydrite increased with increasing pH, as did desorption of arsenate, but MMAs(V) was desorbed in greater quantities than arsenate at any given pH. DMAs(V) was almost completely desorbed from ferrihydrite by phosphate. Desorption trends for arsenate, MMAs(V) and DMAs(V) from goethite were different from those observed for ferrihydrite, but no explanation were given for this phenomenon.

7 Effect of Phosphate on the Removal of Arsenic Coprecipitated with or Sorbed on Metal Oxides

Whereas studies have been carried out on the factors (surface coverage, residence time, pH) which influence the desorption of arsenate previously sorbed onto oxides, phyllosilicates and soils (O'Reilly et al. 2001; Liu et al. 2001; Arai and Sparks 2002; Violante and Pigna 2002; Pigna et al. 2006), scant information are available on the possible desorption of arsenate coprecipitated with iron or aluminum. In natural environments arsenic may form precipitates or coprecipitates with Al, Fe, Mn and Ca. Coprecipitation of arsenic with iron and aluminum are practical and effective treatment processes for removing arsenic from drinking waters and might be as important as sorption to preformed solids.

Recently, studies on the sorption of phosphate on and the removal of arsenate from aluminum-arsenate or iron-arsenate coprecipitates formed at arsenate/aluminum (or iron) molar ratio (R) of 0.1 and pH 4.0, 7.0 or 10.0 have been carried out (Violante et al. 2006, 2007). Figure 10 shows the sorption of phosphate on and the desorption of arsenate from two samples formed at pH 7.0 and R = 0.1, obtained by coprecipitating aluminum and arsenate (7R0.1) or by adding arsenate immediately after the precipitation of aluminum (7AR0.1). These samples, aged 30d at 50°C, showed similar surface area (about 135 $m^2\ g^{-1}$), and mineralogy (presence of poorly crystalline boehmite) but different reactivity. In fact the sorption of phosphate onto 7AR0.1 was more than two times lower that on 7R0.1 (Fig. 10A), whereas greater amounts of arsenate were released from 7AR0.1 than 7R0.1 (Fig. 10B). Evidently, in the 7AR0.1 sample arsenate anions, added to a preformed aluminum precipitate, were sorbed on the external surfaces and occupied many sorption sites and, consequently, prevented the fixation of phosphate more efficiently than 7R0.1 where arsenate anions, being mainly enmeshed in the precipitate, were not easily accessible and not easily desorbed by phosphate. A similar behaviour was ascertained by using iron-arsenate coprecipitates (Violante et al. 2007).

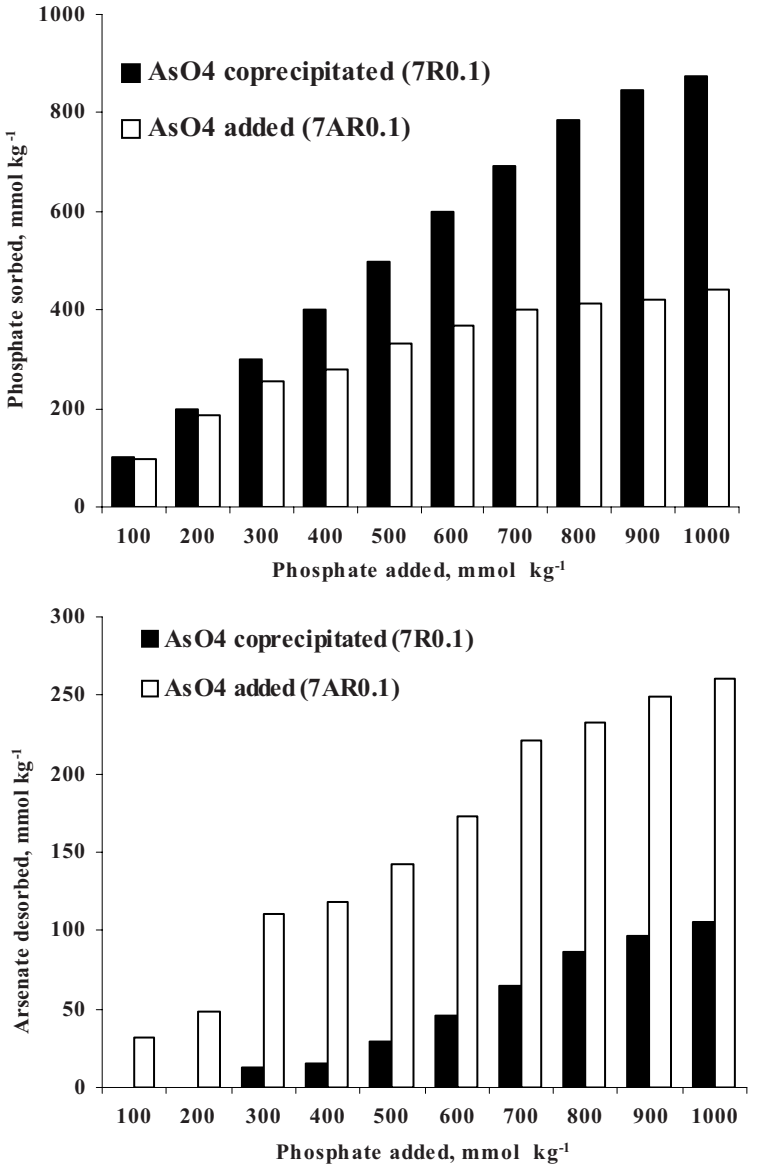

Fig. 10. Sorption of phosphate (PO_4) (A) and desorption of arsenate (AsO_4) (B) from two samples formed at pH 7.0 and R = 0.1, obtained coprecipitating aluminum and arsenate (7R0.1) or by adding arsenate (7AR0.1) immediately after the precipitation of aluminum. Reaction time was 24 hours. Redrawn from Violante et al. (2006).

8 Arsenic Sequential Extraction from Polluted Soils

Although sequential fractionation procedures generally do not allow assessing the precise association of elements with each soil mineralogical phase, they can provide operationally defined phase associations and may be a powerful tool for the identification of some of the main binding sites, allowing to assess the potential for remobilisation and bioavailability of arsenic in polluted soils (Wenzel et al. 2001; Martin et al. 2007a).

The fractionation of the arsenic may be carried out according to the method of Wenzel et al. (2001). Briefly, arsenic is sequentially extracted with: (1) 0.05M K_2SO_4 at 20°C for 4 h; (2) 0.05M KH_2PO_4 at 20°C for 16 h; (3) 0.2M NH_4^+-oxalate buffer in the dark, at pH 3.25 and 20°C for 4 h; (4) 0.2M NH_4^+-oxalate buffer + ascorbic acid at pH 3.25 and at 96°C for 0.5 h and finally (5) HNO_3/H_2O_2 or HCl/HNO_3 hot digestion. The obtained As fractions are defined by these authors as associated to: (1) non-specifically sorbed; (2) specifically-sorbed; (3) bound to amorphous and poorly-crystalline hydrous oxides of Fe and Al; (4) bound to well-crystallized hydrous oxides of Fe and Al; and (5) residual phases. These authors demonstrated that partitioning of arsenic among these fractions in 20 soils was (%, medians and ranges): (1) 0.24 (0.02–3.8); (2) 9.5 (2.6–25); (3) 42.3 (12–73); (4) 29.2 (13–39); and (5) 17.5 (1.1–38).

The arsenic extraction from two polluted Italian soils from Scarlino (Tuscany, Italy) containing high amounts of arsenic (104 mg kg^{-1}, Vetricella soil and 190 mg kg^{-1}, La Botte soil) was studied (Branco 2007). Arsenic was in the most part recovered in the crystalline oxides (about 60–63%; Figs. 11A and B). Another abundant fraction (19–20%) of arsenic was obtained by NH_4-oxalate, which is effective for targeting amorphous Fe and Al oxides (Wenzel et al. 2001). The arsenic fraction extracted with KH_2PO_4 was about 7% for each soils. The fraction not specifically sorbed (easily exchangeable) that form outer-sphere complexes onto the mineral surfaces was very low (< 1%). The scarce residual arsenic fraction (11–13%) suggested a low presence of primary minerals rich in this metalloid (Fig. 11). About 90% of arsenic present in these soils was not available for plants.

Martin et al. (2007a) investigated the accumulation and potential release of arsenic in a paddy field in Bangladesh irrigated with arsenic contaminated groundwater. The oxalate-extractable fraction related to amorphous hydrous oxide-bound arsenic represented the dominant arsenic form in the surface layer (47%). A high percentage of arsenic was removed by phosphate (22%).

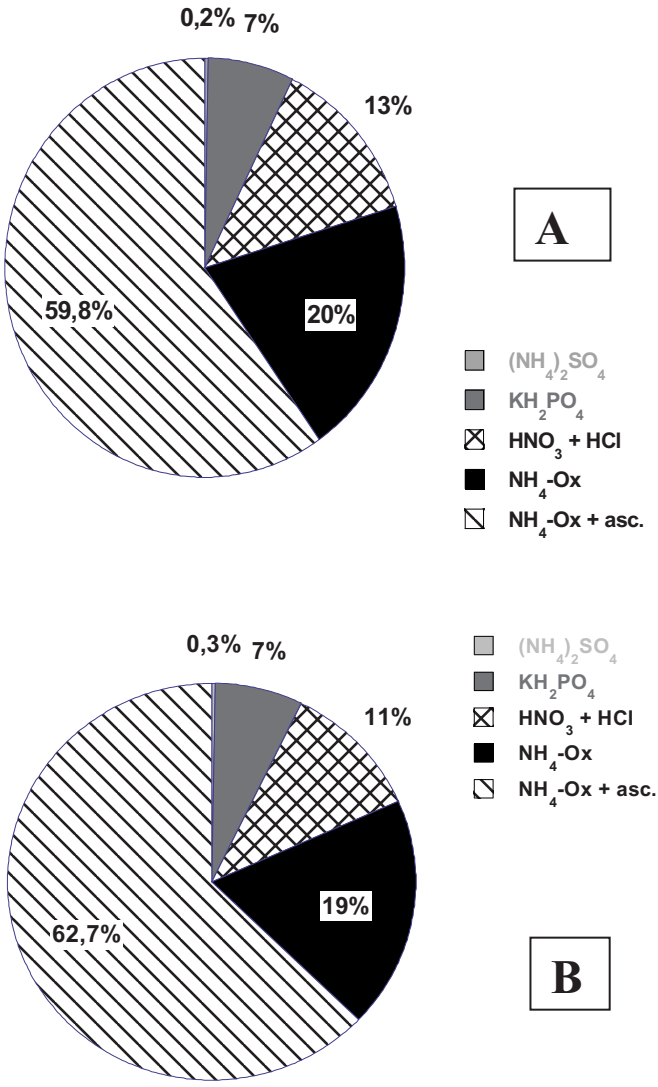

Fig. 11. Arsenate fractionation from two Italian polluted soils. (**A**) La Botte soil containing 190 mmol As kg^{-1}; (**B**) Vetricella soil containing 104 mmol As kg^{-1}. From Branco (2007).

9 Conclusion

Sorption and desorption of arsenic in terrestrial environment is affected by many factors as oxidation state of this element, pH, nature of sorbents, presence of organic and inorganic ligands, surface coverage, time of reaction and residence time of arsenic on the surfaces of the sorbents. Many studies have been carried out on competition in sorption between arsenic (mainly arsenate) in the presence of inorganic and organic anions onto soil components and soils in binary system, but scant experiments have been conducted on the sorption of arsenic in the presence of three or more ligands. Furthermore more information are available on the factors which affect the sorption of arsenic, than the desorption of arsenic. Unfortunately, the effect of organic ligands, both nutrients and $LMMOL_s$ (root exudates or microbial metabolites) on the mobility of arsenite are tremendously poor. The mobility of arsenic present in coprecipitates with Al, Fe, Ca or Mn still needs to be observed. To predict the mobility and potential toxicity of arsenic in natural environments more studies are necessary on the concomitant effects the clay minerals, organic and inorganic ligands, time of reaction and surface coverage have on the sorption/desorption processes of arsenate and (mainly) arsenite.

Acknowledgments

This study was supported by the Italian Research Program of National Interest (PRIN), year 2006. DiSSPA n. 128.

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3 Role of Bacteria and Bacteria-Soil Composites in Metal Biosorption and Remediating Toxic Metal-Contaminated Soil Systems

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1 Introduction

Bioremediation of sites that are contaminated with toxic metals is an important issue in environmental restoration. Bacteria have long been known for their ability to Itake up metals from their immediate environment (Borrok and Fein 2004). The efficiency of bacterial cells in concentrating metals is related to their large surface area-to-volume ratio and high surface density of charge. The cell surfaces of all bacteria are negatively

charged owing to the presence of various anionic structures. *Bacillus subtilis*, for example, has an isoelectric point at pH 2.4, and an average surface charge excess of 1.6 μmol/mg dry biomass over the pH range of 2.4 to 9 (Yee et al. 2004b). Accordingly, bacterial cell walls have a strong affinity for metal cations. Intact bacterial cells, live or dead, and their products are also highly efficient in accumulating both soluble and particulate forms of metals. Bacteria therefore play an important role in the speciation, fate and transport of metals, metalloids and radionuclides in soil and associated environments.

The number of bacteria in soil may reach ~10^9 per gram. Since bacterial communities in soil are composed of many different cells in a matrix of variable-charge exopolysaccharides, their metal binding capacity exceeds that of planktonic cells (Mclean et al. 2002). Furthermore, bacteria in soil live in an ecosystem that is dominated by solid particles. Indeed, 80–90% of the microorganisms in soil are associated with solid surfaces (Nannipieri et al. 2003) through electrostatic interactions, physical adhesion, and covalent bonding (Theng and Orchard 1995). By forming a coat over mineral surfaces bacteria and their extracellular polysaccharides represent a significant fraction of the total surface area of soil that is exposed to the aqueous phase (Daughney et al. 2001).

Bacterial remediation of metal-polluted environments, and the mechanisms underlying metal immobilization, have been the subject of several recent reviews (Stephen and Macnaughton 1999; Gadd 2000; Barkay and Schaefer 2001; Valls and de Lorenzo 2002). There is general agreement that biosorption is an emerging technology capable of removing very low levels of toxic metals. Here we focus on the sorption of toxic metals by bacteria with special reference to surface binding and complexation. The bioaccumulation characteristics of bacteria-soil composites will also be discussed.

2 Mechanisms of Metal Sorption by Bacteria

As shown in Fig. 1 biosorption comprises a variety of processes including ion exchange, chelation, adsorption, and diffusion through cell walls and membranes all of which are dependent on the species used, the biomass origin and, and solution chemistry (Gavrilescu 2004). Biosorption is a fast and reversible process for removing toxic metal ions from solution.

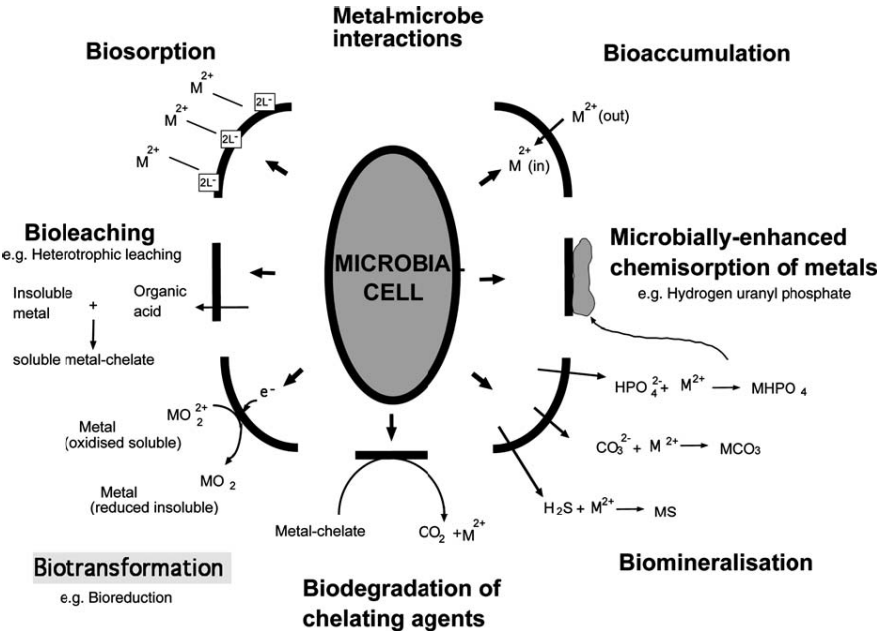

Fig. 1. Schematic diagram of the different processes involved in the metal-microbe interaction (from Tabak et al. 2005).

McEldowney (2000) reported that 65% of Cd^{2+} was associated with the cell walls of *Pseudomonas fluorescens*, while 33% was present in the cytoplasm, and 2% was bound to extracellular polymeric substances (EPS) excreted by the bacteria. EPS include polyssacharides, proteins and siderophores. Organic matter, derived from dead microbes, can also form extracellular complexes with metals.

The large propensity of gram-positive bacteria for sorbing metal cations is due to the high concentration in their cell walls of peptidoglycan and teichoic acid polymers that contain numerous negatively charged functional groups. The cell walls of gram-negative bacteria, on the other hand, are low in these polymeric compounds, and hence show a limited capacity for metal sorption (Beveridge 1989). In general, bacterial cell walls behave like polyelectrolytes and interact with ions in solution so as to maintain electroneutrality. Thus, the principal mechanism by which metal ions interact with bacterial cell surfaces is through electrostatic attraction, supplemented by van der Waals forces, covalent bonding, redox interactions, and extracellular precipitation.

An electron microscopy study by Mullen et al. (1989) showed that Cd^{2+}, Cu^{2+} and La^{3+} accumulated on the cell surface of *Bacillus cereus, B. subtilis, E. coli* and *Pseudomonas aeruginosa* as needle-like, crystalline precipitates, while Ag^{+} precipitated as discrete colloidal aggregates at the cell surface and occasionally in the cytoplasm. The needle-like and hexagonal precipitates were also found for the biosorption of Ni^{2+} on the cell surface of *P. fluorescens* 4F39 at pH 9 and it was suggested as a microprecipitation process that followed on ion exchange (Lopez et al. 2000).

2.1 Functional Groups

Bacterial cell walls contain different types of negatively charged (proton-active) functional groups, such as carboxyl, hydroxyl and phosphoryl that can adsorb metal cations, and retain them by mineral nucleation. Reversed titration studies on live, inactive *Shewanella putrefaciens* indicate that the pH-buffering properties of these bacteria arise from the equilibrium ionization of three discrete populations of carboxyl (pKa = 5.16 ± 0.04), phosphoryl (pKa = 7.22 ± 0.15), and amine (pKa = 10.04 ± 0.67) groups (Haas et al. 2001). These functional groups control the sorption and binding of toxic metals on bacterial cell surfaces.

Despite differences in cell wall structure and composition, *B. subtilis* and *E. coli* show a remarkably similar behavior with respect to the sorption of Cd^{2+} and Pb^{2+}. This observation is explained in terms of the specific chemical reactivity of acidic functional groups (e.g., carboxyl, phosphoryl) on the cell wall of both bacteria (Kulczycki et al. 2002). UO_2^{2+} was reported to exclusively bound to phosphoryl functional groups of *B. subtilis a*t pH 1.67. The average distance between the U and P atoms was 3.64 ± 0.01 Å. This value indicated formation of an inner-sphere complex with an oxygen atom shared between UO_2^{2+} and a phosphoryl ligand. With increasing pH (3.22 and 4.80), UO_2^{2+} was increasingly bound to carboxyl functional groups giving an average distance of 2.89 ± 0.02 Å between the U and the C atoms. This U-C distance was also indicative of an inner-sphere complex with two oxygen atoms shared between UO_2^{2+} and a carboxyl ligand (Kelly et al. 2002). Likewise, Nikovskaya et al. (2002) confirmed the binding of U to carboxyl groups on the cell surface of *Bacillus cereus*. The study by Boyanov et al. (2003) showed that Cd on the cell wall of gram-positive *B. subtilis* was predominantly bound to phosphoryl ligands below pH 4.4, while at higher pH binding to carboxyl groups became increasingly important. At pH 7.8, the activation of an additional binding site was ascribed to a phosphoryl group although the Cd-P distance was smaller than that found at low pH. The molecular speciation of

Zn within the biofilm of *Pseudomonas putida* examined with Zn K-edge extended X-ray absorption fine structure (EXAFS) spectroscopy confirmed the importance of phosphoryl functional groups in Zn sorption at neutral pH (6.9). Zinc sorption to the biofilm was attributed to predominantly Zn-phosphoryl (85±10 mol %) complexes, with a smaller contribution to sorption from carboxyl-type complexes (23±10 mol %) (Toner et al. 2005). By using synchrotron radiation Fourier transform infrared spectroscopy along with potentiometric titration and metal sorption experiments, Yee et al. (2004a) proposed that, on the cyanobacterial cell wall, the carboxyl groups are the dominant sink for metals such as Cu^{2+}, Cd^{2+}, and Pb^{2+} at near neutral pH. Based on thermodynamic modeling, Gorman-Lewis, et al. (2005) reported that uranyl hydroxide, uranyl-carbonate, and calcium-uranylcarbonate species each can form stable surface complexes on the cell wall of *B. subtilis* from pH 1.5 to 9. The binding of metal ions with bacterial functional groups may be influenced by the cell/metal ratios. At high bacteria-to-Cd ratios, Cd adsorption occurs by formation of a 1:1 complex with deprotonated cell wall carboxyl functional groups for a thermophile bacertium *Anoxybacillus flavithermus*. At lower bacteria-to-Cd ratios, a second adsorption mechanism occurs at pH > 7, which may correspond to the formation of a Cd–phosphoryl, CdOH–carboxyl, or CdOH–phosphoryl surface complex (Burnett et al. 2006).

2.2 Environmental Factors Affecting Metal Biosorption

Many environmental factors influence the chemical reactivity of the binding sites on bacterial cell surfaces and the subsequent biosorption of metals. These factors include pH, ionic strength, temperature, and the presence of other metals and organic compounds. The binding of metals by bacteria is also affected by nutrient and oxygen levels. The capacity of indigenous anaerobic sulphate-reducing bacteria for accumulating radioactive elements, for example, can be enhanced by adjusting the levels of such essential factors as water, oxygen, and nutrients in the soil (Groudev et al. 2001). Limited biosorption of Cd was observed observed when the bacteria were incubated with a “poor” soil extract medium, while biosorption was relatively high in a “rich” incubating medium (Lebeau et al. 2002).

2.2.1 Growth Phase

The ability of bacteria to accumulate toxic metals also varies with cell age. Shuttleworth and Unz (1993) reported that one-day-old cells of *Thiothrix* strain A1 accumulated considerably less Ni or Zn than its 2–5 day old

counterparts. However, the biosorptive capacity of dead cells may be greater, equivalent to, or less than that of living cells (Kurek et al. 1982; Ledin 2000; Huang et al. unpublished data). For example, biosorption of Cr by the dead cells of *Bacillus circulans* and *Bacillus megaterium* was higher than by living cells (Srinath et al. 2002).

Using suspensions of *B. subtilis* cultured to the exponential, stationary, and sporulated phase, Daughney et al. (2001) measured the effect of growth phase on surface site concentrations, deprotonation constants, and metal-binding constants by acid-base titration and Cd batch sorption. The concentrations and p*K*a values of deprotonated sites decreased as the cells moved from an exponential to a stationary phase, but remained constant during transition from a stationary to a sporulated phase. Due to variations in site concentrations and deprotonation constants, Cd binding constants were largest for stationary-phase cells and smallest for sporulated cells, even though the former sorbed 5–10% less metal than the exponential-phase cells, and 10–20% more metal than sporulated cells. These results suggest that any attempt at predicting proton or metal sorption by bacteria must consider the growth phase of the population. In a recent study, Yilmaz and Ensari (2005) showed that the maximum uptake of Cd by *B. Circulans* EB1 occurred during the mid-logarithmic phase of growth. The sorption capacity of resting cells was markedly higher than that of growing cells. Nevertheless, Borrok et al. (2004b) found that the mass-normalized extent of Co sorption by both *P. fluorescens* and *Shewanella oneidensis* MR-1 was independent of growth conditions although bacterial cell size changed markedly in response to nutrient and oxygen concentrations.

2.2.2 pH

Accumulation of metals by bacteria may be influenced by pH. Simine et al. (1998) reported that the biosorptive capacity of Pb by *Brevibacterium sp* was sensitive to pH. Competitive cation sorption experiments by Fowle and Fein (1999), using both single and double bacteria systems with two *Bacillus* strains, showed little uptake at pH < 4. However, the extent of Cu, Pb and Cd sorption increased due to the enhanced deprotonation of surface functional groups at pH > 4. A pH-dependent accumulation of heavy metals by *P. fluorescens* 4F39 was also observed by Lopez et al. (2000). Similarly, the sorption of Cd^{2+} and Pb^{2+} by *B. subtilis* and *E. coli* was strongly dependent on pH from 4.2 to 5.6 (Kulczycki et al. 2002). A number of studies on U revealed that specific pH ranges favor the biosorption. Haas et al. (2001) reported that U(VI) could form a surface complex with the cells of *S. putrefaciens* in the pH 2–8 interval, with maximum adsorption occurring at pH ~5. The sorption of UO_2^{2+} by *B. subtilis* at pH < 3.0 was

independent of pH and this was ascribed to the interaction between uranyl cations and neutrally charged phosphoryl groups on the cell wall (Kelly et al., 2002). The sorption of U(VI) by negatively charged *B. cereus* was maximal at pH 4.2–4.5, when U(VI) was present in the form of positively charged hydroxocomplexes. However, there was minimal interaction between U(VI) and *B. cereus* cells at pH 8 when U(VI) formed negatively charged water-soluble hydroxocarbonate complexes. The sorption of U(VI) in a weakly acidic medium was not affected by the presence of Co, Sr, Cu, Ca, Mg, and Zn ions. These ions, however, inhibited sorption under neutral pH conditions (Nikovskaya et al. 2002).

The pH-dependence of metal sorption by bacteria can vary significantly with metal concentration. For example, the sorption of Pb by *Pseudomonas atlantica* at the highest metal concentration (5×10^{-6} M) was almost independent of pH, whereas sorption markedly increased with pH when the metal concentration was low (Ledin 2000). On the other hand, the accumulation of Cd by *Pseudomonas cepacia* showed an increased pH dependence with increasing metal concentration (Savvaidis et al. 1992). The pH dependence may also be influenced by ionic strength.

2.2.3 Ionic Strength

The sorption of metals by bacteria is often influenced by the presence of various cations. Small et al. (2001), for example, reported that the sorption of Sr^{2+} to *Shewanella alga* was strongly dependent on ionic strength. With increasing ionic strength the apparent surface complex formation constant for *S. alga* increased from $100^{-0.51}$ to $10^{-0.26}$, suggesting that only high affinity sites remained to bind Sr^{2+} at high ionic strength. Since the sorption of Sr(II) and Ba(II) by *B. subtilis* showed a strong ionic strength dependence, Yee et al. (2004b) proposed that these metal ions were bound to the bacterial cell wall as an outer-sphere complex.

The sorption of U (VI) by *S. putrefaciens* was somewhat sensitive to ionic strength (NaCl) in the range 0.02–0.10 M. The ionic strength dependence was similar to that measured for metal-oxide surfaces and Gram-positive bacteria, and appeared to be similarly controlled by competitive speciation constraints (Haas et al. 2001).The sorption of thorium by bacteria, in terms of amount and time course, was almost unaffected by coexisting uranium. However, the process was accelerated when it occurred after the adsorption of uranium. The rate of thorium adsorption was also enhanced when uranium was added after thorium was adsorbed (Tsuruta 2004). Alterations in the structural and chemical properties of bacterial cell surfaces resulting from exposure to acidic solutions may affect cation binding. Borrok et al. (2004b), for example, observed that bacteria such as

P. mendocina, P. aeruginosa, B. subtilis, and *B. cereus* adsorbed more Cd, Co, and Pb after exposure to acidic solutions than the corresponding unexposed cells. The increase in sorption following acid treatment was attributed to the irreversible displacement of structurally bound Mg and Ca by protons. The protonated sites can participate in reversible metal sorption reactions.

2.2.4 Metal Type

Mullen et al. (1989) found that the affinity of *B. cereus, B. subtilis, E. Coli* and *P. aeruginosa* for metal cations decreased in the order Ag > La > Cu > Cd, while Lopez et al. (2000) reported the following order: Ni >> Hg > U >> As > Cu > Cd > Co > Cr > Pb. According to their sorption behavior, metal cations may be grouped into two types. The accumulation of Type I metal cations (Ni, Cu, Pb, Cd, Co) increases as pH increases, reaching a maximum at the pH before precipitation, occurs, while the maximum accumulation of Type II metal cations (Cr, As, U, Hg) is not associated with precipitation. Lead could compete with Cd for attachment to bacterial surface sites when a solution containing both metals was added (Simine et al. 1998). Lead removal occurred by a combination of fast physico-chemical adsorption and slow accumulation mediated by cell metabolism. Savvaidis et al. (2003) found that the biosorption of Cu by *P. cepacia* was dependent on the concentration of added Cu. Copper uptake by the cells was rapid over the range of copper concentrations tested and complete within the first 10 min of incubation time. Copper uptake by *P. cepacia* cells apparently involved surface binding and not intracellular accumulation by active transport.

2.2.5 Organic Ligands

Organic ligands may affect the adsorption and binding of metals on bacteria by forming chelates. Fein and Delea (1999) reported that aqueous EDTA competed strongly with the bacterial surface for Cd ions, markedly reducing Cd sorption by *B. subtilis*. Similarly, the presence of humic acid (HA) diminished Cd sorption by the surface of *B. subtilis* (Wightman and Fein 2001). This was attributed to formation of an aqueous Cd-humate complex under moderate to high pH conditions. However, the solubility of HA was apparently unaffected by the presence of aqueous Cd.

The removal of Pb by *Brevibacterium sp* strain PBZ was markedly enhanced by the presence of glucose (Simine et al. 1998). Desorption of the metal by EDTA restored the binding capacity of the cells. U(VI) could be desorped from the cell surface of *B. cereus* by citric acid or sodium bicarbonate with the formation of water-soluble complexes although U(VI) was strongly bound on the cell surface of the bacteria. However, uranyl in

the form of organic complexes with citric, humic, and fulvic acids was not sorbed by biocolloids (Nikovskaya et al. 2002). A study with metabolic inhibitors on biosorption revealed that the sorption of copper by *P. cepacia* was not affected by cyanide and azide (Savvaidis et al. 2003).

3 Metal Tolerance and Sorption Capacity of Bacteria

A large variety of bacterial strains have been isolated from toxic metal-contaminated environments. These strains may be useful for the investigation and understanding of the tolerant and adsorptive mechanisms of bacteria for toxic metals. Yilmaz (2003) screened a heavy metal-resistant *Bacillus sp.* strain EB1 from a contaminated soil in southeast Turkey. This strain exhibited high minimal inhibitory concentration (MIC) values for metals and was capable of removing 90% of Mn, 68% of Zn, 65% of Cu, 45% of Ni and 40% of Co during its active growth cycle with a specific biosorption capacity of 25, 22, 20, 13 and 12 mg L^{-1}, respectively. Since the cells could grow in the presence of significant concentrations of metals and have a high metal biosorption capacity under aerobic conditions, this *Bacillus* sp is potentially useful for the in-situ bioremediation of heavy metal-contaminated aqueous systems. Subsequently, Yilmaz and Ensari (2005) were able to show that *B. circulans* EB1 had a high tolerance to Cd as well as a high sorption capacity for this metal. When grown in the presence of 28.1 mg Cd L^{-1} this bacterium could sorb 5.8 mg Cd g^{-1} dry wt biomass during the first 8 h. After preconditioning with low concentrations of Cd, the sorption capacity of the cells increased to 6.7 mg Cd g^{-1}. Since both the resting and growing cells had a high sorption capacity for Cd, *B. circulans* EB1 could serve as an excellent biosorbent for Cd in natural environments.

Mullen et al. (1989) reported that *Bacillus cereus, B. subtilis, E. coli* and *P. aeruginosa* were able to sorb an average of 89% of the total Ag^{+} and 12–27% of the total Cd^{2+}, Cu^{2+} and La^{3+} from a 1mM solution. Using polyacrylamide-entrapped cells of *Brevibacterium sp* strain PBZ, Simine et al. (1998) measured a sorption capacity of ~40 mg g^{-1} and ~13 mg g^{-1} dry biomass for Pb and Cd, respectively. Hall et al. (2001) isolated two bacterial strains of *P. syringae* that were tolerant to 1000 mg L^{-1} Cu. Similarly, Amoroso et al. (2001) were able to obtain *Streptomyces spp*. strains R22 and R25 with a high tolerance to Cr from sediments of the Salí River, Argentina. The cells of R22 and R25 could accumulate 10.0 and 5.6 mg Cr g^{-1} dry weight, respectively, from a concentration of 50 mg Cr mL^{-1}. Cell fractionation studies with strain R22 showed that most of the chromium

was associated with the cell walls. Srinath et al. (2002) isolated two species of bacteria (*B. circulans* and *Bacillus megaterium*) from a treated tannery effluent. The cells could accumulate 32–35 mg Cr(VI) g^{-1} dry weight from an initial concentration of 50 mg Cr(VI) L^{-1}, decreasing the residual concentration of Cr(VI) to the permissible limit within 24 h. The sorption capacity of *B. subtilis* for Cd^{2+} and Pb^{2+} was 0.36 mmol g^{-1} and 0.27 mmol g^{-1}, respectively, while the corresponding values for *E. coli* were 0.10 mmol g^{-1} and 0.21 mmol g^{-1} (Kulczycki et al. 2002).

Using a multi-compartment system (Partitioning in Geobiochemical Systems, PIGS), Ledin et al. (1996) compared the accumulation of metals by various soil components (a bacterium, a fungus, peat, a clay and aluminum oxide) with or without the presence of fulvic acid. Although making up only a minor part of the solid phase, microorganisms made a substantial contribution to metal accumulation. Geochemical modeling using the Langmuir equation indicated that *Shewanella alga* and *Shewanella putrefaciens* sorbed significantly greater quantities of Sr^{2+} than hydrous ferric oxide (Small et al. 1999).

Groudev et al. (2001) isolated indigenous anaerobic sulphate-reducing bacteria from agricultural soils in southeastern Bulgaria that have been contaminated with U, Ra, Th, Cu, Cd and Pb from mining and mineral processing of polymetallic ores. These bactaeria were efficient in immobilizing radioactive elements and heavy metals under field conditions, reducing their concentrations in the soil to below their respective permissible levels within eight months. Cultures of *Desulfovibrio desulfuricans*, *Desulfotomaculum gibsoniae*, and *Desulfomicrobium hypogeia* were capable of removing 99.99% of the soluble Co^{2+} when $CoCl_2$ was used with no chelating agents. The same cultures and *Desulfoarcula baarsi* removed 98–99.94% of soluble Co(II) when the metal was complexed with nitrilotriacetate (Co-NTA) (Krumholz et al. 2003).

Tsuruta (2004) reported that strains of the gram-positive bacteria *Arthrobacter nicotianae* IAM12342, *B. megaterium* IAM1166, *B. Subtilis* IAM1026, *Micrococcus luteus* IAM1056, *Rhodococcus erythropolis* IAM1399, and *Streptomyces levoris* HUT6156 had a high capacity for sorbing thorium, while *S. albus* HUT6047, *S. levoris* HUT6156, and *A. nicotianae* IAM12342 were efficient in sorbing uranium. The most efficient among these microorganisms was *S. levoris* which could sorb about 383 μmol thorium and 390 μmol uranium per gram dry weight of cells from a thorium or uranium solution at pH 3.5. Huang et al. (2005) isolated a bacterial strain, *Enterobacter aerogenes* NTG-01, from a heavily Cu-contaminated soil in the mining area near Daye, Hubei province, China. This strain was tolerant to 3 mM Cu and 3 mM Cd. It also showed great potential for sorbing Cu and Cd ions.

Some heavy metal-tolerant bacterial strains and their sorption capacities for Cu and Cd are listed in Table 1. These bacteria show great potential for remediating soils that are contaminated with toxic metals. Our pot culture experiments showed that the growth of tobacco plants in a Cd-polluted Yellow Brown Soil (Alfisol) was significantly promoted by inoculating the soil with *P. Putida* in comparison with the non-inoculated soil (Fig. 2).

Table 1. Sorption capacity (mmol kg^{-1}) of some bacterial species for Cu and Cd

Bacteria	Cu	Cd	Reference
Bacillus subtilis	173	—	Mayers and Beveridge (1989)
Brevibacterium sp.	540	140	Vecchio et al. (1998)
Bacillus sp. (spores)	829	—	He and Tebo (1998)
Pseudonmonas aeruginosa	213~222	—	Langley and Beveridge (1999)
Thiobacillus ferrooxidans	1859	—	Ruiz-Manríquez et al. (1997)
Zooglea ramigera	—	13	Scott and Palmer (1988)
Gram negative bacteria	—	120	Gourdon et al. (1990)
B. Licheniformis	—	1274	Zouboulis et al. (2004)
Enterobacter aerogenes NTG-01	395	171	Huang et al. (2005)
Pseudomonas putida	206	598	Huang et al. (to be published)
Spirulina platensis 439	1992	2347	Huang et al. (to be published)

4 Chemical Modeling of Metal Biosorption

The Langmuir and Freundlich equations have often been employed to model the sorption of metal ions by bacteria. Mullen et al. (1989) used the Freundlich isotherm to describe the sorption of Cd and Cu by *B. cereus, B. subtilis, E. coli* and *P. aeruginosa* over the concentration range of 0.001–1mM. The respective values of the Freundlich constant (Kf) indicated that *E. coli* was most efficient at sorbing Cd^{2+} and Cu^{2+}.

Hall et al. (2001) measured the biosorption of copper by *P. syringae*, fitting the experimental data to the Freundlich, Brunauer-Emmett-Teller (BET), and Langmuir equations. Meaningful maximum sorption capacities

and sorption affinity coefficients were derived from the Langmuir equation. The results were explained in terms of sorbate/sorbent interactions involving a passive mechanism by which copper was largely associated with the outer cell wall. Used acid–base titrations, Daughney et al. (1998) determined the concentrations and deprotonation constants of specific functional groups on the surface of *B. licheniformis*. The carboxyl, phosphate and hydroxyl surface functional groups had pKa values of 5.2, 7.5 and 10.2, respectively, while the average values for the Cd-, Pb-, Cu- and Al–carboxyl stability constants (log *K*) were 3.9, 4.6, 4.9 and 5.8, respectively. The results indicate that *B. subtilis* or *B. licheniformis* have different relative and absolute concentrations of surface sites and slightly different deprotonation and metal adsorption stability constants. Acid–base titration of suspensions containing *B. subtilis* or *B. licheniformis* in 0.01 and 0.1 M $NaNO_3$ indicated that the constant capacitance model provided the best description of the experimental data (Daughney and Fein 1998). The model parameters varied between independently grown bacterial cultures, possibly because of cell wall variations arising from genetic exchange during

Fig. 2. Remediation of Cd^{2+} toxicity by inoculation of *P. putida* and its effect on plant growth. (**A**) 15 day-old seedlings of tobacco (*Nicotiana bentamiana*) grown for 40 days in soils containing 150 μg Cd^{2+} kg^{-1}; (**B**) same as A but inoculated with 10^8 cells of *P. putida* g^{-1}, (**C**) control soil (not inoculated, but mixed with the same medium) (authors' unpublished data, 2006).

reproduction. Sorption of Cd, Pb, and Cu by *B. subtilis* and *B. licheniformis* showed that the stability constants varied substantially but systematically between the two bacterial species at the two different ionic strengths. The dependence of sorption on ionic strength, shown by *S. alga*, was consistent with the formation of outer-sphere complexes in the diffuse double layer (Small et al. 2001).

Fowle and Fein (1999) measured the sorption of Cd, Cu, and Pb by *B. subtilis* and *B. licheniformis* using the batch technique with single or mixed metals and one or both bacterial species. The sorption parameters estimated from the model were in excellent agreement with those measured experimentally, indicating that chemical equilibrium modeling of aqueous metal sorption by bacterial surfaces could accurately predict the distribution of metals in complex multicomponent systems. Fein and Delea (1999) also tested the applicability of a chemical equilibrium approach to describing aqueous and surface complexation reactions in a Cd-EDTA-*B. subtilis* system. The experimental values were consistent with those derived from chemical modeling.

In order to test the reversibility of metal-bacteria interactions, Fowle and Fein (2000) compared the extent of desorption estimated from surface complexation modeling with that obtained from sorption-desorption experiments. Using *B. subtilis* these workers found that both sorption and desorption of Cd occurred rapidly, and the desorption kinetics were independent of sorption contact time. Steady-state conditions were attained within 2 h for all sorption reactions, and within 1 h for all desorption reactions. The extent of sorption or desorption remained constant for at least 24 h and up to 80 h for Cd. The observed extent of desorption in the experimental systems was in accordance with the amount estimated from a surface complexation model based on independently conducted adsorption experiments.

Fein et al. (2001) used a linear free-energy approach to compare previously measured stability constants for *B. subtilis* metal-carboxyl surface complexes with aqueous metal-organic acid anion stability constants. The organic acids are acetic, oxalic, citric, and tiron. The sorption behavior of Co, Nd, Ni, and Sr was well described by considering metal-carboxyl surface complexation only. In the case of Zn, however, complexation with both carboxyl and phosphoryl groups was required to attain a suitable fit to the data. The best correlation between the stability constants of bacterial surface carboxyl complexes and those of aqueous organic acid anion complexes was obtained for metal-acetate aqueous complexes, with a linear correlation coefficient of 0.97. This correlation applies only to unhydrolyzed aqueous cations complexed to surface carboxyl groups. It does not hold for the binding of metals to other functional groups on the bacterial

surface. Nevertheless, the observed correlation allows the aqueous metal-carboxyl site complexation to be estimated for a wide range of metal cations for which experimental data are absent. This technique together with observations of metal complexation involving a range of bacterial species (Yee and Fein 2001), provide insight into the effect of biosorption on metal mobility in soil and geologic environments .

Haas et al. (2001) have investigated the sorption of U(VI) by *Shewanella putrefaciens,* a Gram-negative, facultatively anaerobic bacterium, using acid-base potentiometric titration. Taking a bacterial specific surface area of 55 m^2/g the site densities for carboxyl, phosphoryl and amine groups on the bacterial surface were estimated at 31.7 μmol sites/g bacteria (0.35 ± 0.02 sites nm^{-2}), 8.95 μmol/g (0.11±0.007 sites nm^{-2}), and 38.0 μmol/g (0.42±0.008 sites nm^{-2}), respectively. The sorption results were explained in terms of the formation of two types of surface complexes: $\equiv COO–UO_2^+$ and $\equiv PO_4H–UO_2(OH)_2$. The geochemical speciation models could be extended to bacteria that are capable of precipitating a wide variety of environmentally important metals and metallic species.

Fowle et al. (2000) have measured the sorption by a soil bacterium (*B. subtilis*) of uranyl in 0.1 M $NaClO_4$ at 25ºC as a function of pH, time, and solid: solute ratio, using a batch technique. The stoichiometry and thermodynamic stability of the important uranyl-surface complexes indicated that uranyl formed two different surface complexes, one involving neutral phosphate functional groups, and another with deprotonated carboxyl functional groups, on the bacterial cell wall:

$R\text{-}POH^0 + UO_2^{2+} \Leftrightarrow R\text{-}POH\text{-}UO_2^{2+}$ ($\log K = 11.8 \pm 0.2$)

$R\text{-}COO^- + UO_2^{2+} \Leftrightarrow R\text{-}COO\text{-}UO_2^{2+}$ ($\log K = 5.4 \pm 0.2$)

Batch adsorption experiments by Yee and Fein (2002) using aqueous Cd, *B. subtilis*, and quartz as a function of pH showed that the thermodynamic stability constants, determined from binary systems, could successfully describe the distribution of Cd between the aqueous phase and the bacterial and mineral surfaces. The constants could also be used to estimate the distribution of mass in systems, and construct a surface complexation model.

The sorption data of Cd^{2+} and Pb^{2+} by *B. subtilis* and *E. coli* were well described by a one-site complexation model ($r^2 > 0.9$) with Cd^{2+} showing somewhat lower sorption affinities than Pb^{2+} (Kulczycki et al. 2002). A two-site sorption model yielded an improved fit but only for the *E. coli* data. The stability constants for the high- and low-affinity sorption sites differed by several orders of magnitude. The total metal sorption capacity of *E. coli* increased, and moved closer to the value of *B. subtilis* when the presence of low-affinity sorption sites was allowed. Ngwenya et al. (2003) used potentiometric titrations to assess the different types of sites present

on the cell walls of the genus *Enterobacteriaceae*. Stability constants for the sorption of Pb, Cu and Zn by specific sites were determined from batch experiments at different pH values and a constant metal concentration. Three distinct acidic surface sites were indicated having pK values of 4.3 ± 0.2, 6.9 ± 0.5, and 8.9 ± 0.5 corresponding to carboxyl, phosphate and hydroxyl/amine groups with surface densities of $5.0 \pm 0.7 \times 10^{-4}$, $2.2 \pm 0.6 \times 10^{-4}$ and $5.5 \pm 2.2 \times 10^{-4}$ mol g^{-1} dry bacteria, respectively. Only the carboxyl and phosphoryl groups were involved in metal uptake, yielding the following intrinsic stability constants: Log $K_{carboxyl}$ = 3.3 ± 0.1 for Zn, 3.9 ± 0.8 for Pb, and 4.4 ± 0.2 for Cu, while Log $K_{phosphoryl}$ = 5.1 ± 0.1 for Zn and 5.0 ± 0.9 for Pb.

The data of Loukidou et al. (2004) for the equilibrium biosorption of chromium (VI) by *Aeromonas caviae* particles were well described by the Langmuir and Freundlich isotherms. Sorption rates estimated from 'pseudo second-order' kinetics were in satisfactory agreement with experimental data. The results of XAFS study on the sorption of Cd by *B. subtilis* were generally in accord with existing surface complexation models (Boyanov et al. 2003). Intrinsic metal sorption constants were obtained by correcting the apparent sorption constants by the Boltzmann factor. A 1:2 metal-ligand stoichiometry provides the best fit to the experimental data with log *K* values of 6.0 ± 0.2 for Sr(II) and 6.2 ± 0.2 for Ba(II).

Electrophoretic mobility measurements of *B. subtilis* cells with sorbed Sr(II) and Ba(II) supported the 1:2 metal-ligand stoichiometry. Thus, the electrical potential parameters derived from the Donnan model can be used to predict metal binding by bacterial surfaces over a wide range of pH and ionic strength conditions (Yee et al. 2004b). Thermodynamic modeling by Borrok et al. (2004b) further suggests that the concentration of functional groups on bacterial surfaces can increase by as much as five times in response to acid washing, assuming that the stability constants for bacterial surface complexes remain the same. Based on their 2-site non-electrostatic Cd adsorption model, Gorman-Lewis et al. (2006) measured the bulk heats of Cd adsorption onto *B. subtilis* at 25.0°C by titration calorimetry. The bulk Cd enthalpy data yielded the following site-specific enthalpies of Cd adsorption onto bacterial surface Sites 2 and 3, respectively: 0.2 ± 0.4 and +14.4 ± 0.9 kJ mol^{-1}, and the following third law entropies of Cd adsorption onto Sites 2 and 3, respectively: +57 ± 4 and +128 ± 5 J mol^{-1} K. The calculated enthalpies of Cd adsorption are typical of those associated with Cd complexation with anionic oxygen ligands, and the entropies suggest the inner sphere complexation by multiple ligands. The new thermodynamic data enabled quantitative estimates of the temperature dependence of Cd adsorption on bacterial surfaces.

Borrok et al. (2004a) used potentiometric titration to measure Cd sorption by different bacterial consortia, and a surface complexation approach to determine thermodynamic stability constants. When the data were modeled by adopting a single set of stability constants, a similar sorption behavior was shown by a wide range of bacterial species. Further, current models that rely on pure strains of laboratory-cultivated bacterial species appear to overestimate the extent of metal biosorption in natural systems.

Borrok and Fein (2004) employed thermodynamic equilibrium constants from the literature to construct an internally consistent model for the binding of Cd by dissolved humic substances (HS). The binding constants and site density values were directly compared with published data for Cd binding by natural consortia of bacteria. These constants were then combined into a unified model that could account for the competition between bacterial surfaces and dissolved HS as well as their relative contributions to Cd complexation in natural settings. These workers also performed calculations for three representative systems with different concentration ratios of bacteria to HS. The results indicated that the number of available binding sites (per gram) in dissolved HS were two orders of magnitude larger than that associated with bacterial surfaces. HS also had a greater affinity than bacterial surfaces for binding Cd at neutral and near-neutral pH. The combined model further showed that, depending on their relative concentrations, both Cd-humic and Cd-bacteria complexes controlled Cd-speciation in specific natural environments. This modeling approach is useful in that it can easily be extended to other metals and binding ligands; however, appropriate thermodynamic data must be gathered to facilitate the modeling of more realistic systems.

Loukidou et al. (2005) fitted the data for the equilibrium sorption of Cd from aqueous solutions by *Aeromonas caviae* to the Langmuir and Freundlich isotherms. They also conducted, a detailed analysis of sorption rates to validate several kinetic models. A suitable kinetic equation was derived, assuming that biosorption is chemically controlled. The so-called ‘pseudo second-order’ rate expression could satisfactorily describe the experimental data. The adsorption data of Zn on soil bacterium *Pseudomonas putida* were fit with the van Bemmelen-Freundlich model (Toner et al. 2005).

Molecular simulation methods can be a complement to surface complexation modeling on metal–bacteria adsorption reactions, which provides a more detailed and atomistic information of how metal cations interact with specific functional groups within bacterial cell wall. Johnson et al., (2006) applied molecular dynamics (MD) simulations to analyze equilibrium structures, coordination bond distances of metal-ligand complexes.

The adsorption of Cd and Pb onto peptidoglycan and teichoic acid components of the bacterial cell wall was investigated using classical energy force field methods. The different components of the cell wall and their relative binding energies and structural configurations were determined based on the Cerius2 modeling software, energy minimization, conformational analysis, and molecular dynamics in the absence and presence of metals. Force field-based simulation techniques can adequately describe the coordinations and binding distances of Cd ion on the cell wall. However, the classical force field approach failed to depict the observed Pb–cell wall interactions due to possible limitations in the force field parameters, the tendency for Pb to form hydroxides at circumneutral pH, or the major contribution of other adsorption mechanisms.

5 Immobilization of Metals by Bacteria-Soil Composites

Bacteria in soil occur as single cells or multicell colonies and are closely associated with soil particle (mineral) surfaces, forming bacteria-soil composites. Indeed, bacteria and minerals in soil are so intertwined that one often cannot exist without the other. Bacteria-soil composites, therefore, play an important role in the sorption and binding of toxic metals (Fein et al. 1997, Langley and Beveridge 1999, Chenu and Stotzky 2002). Microbial biofilms are present in soils, sediments, and natural waters. They contain bioorganic metal-complexing functional groups and are thought to play an important role in metal cycling in natural and contaminated environments (Toner et al. 2005).

Flemming et al. (1990) reported that isolated *B. subtilis* 168 cell walls, *E. coli* K-12 cell envelopes, and their respective composites with clay minerals were capable of binding appreciable quantities of Ag(I), Cu(II), and Cr(III). However, the envelope-clay and wall-clay mixtures bound less metal than equal amounts of the individual components on a dry-weight basis because the adsorption of the wall or envelope to clay masked or neutralized chemically reactive sites normally available to metal ions (Walker et al. 1989). The retention of heavy metals such as Pb by bacteria-mineral composites under a wide range of environmental conditions was attributed to their large sorption capacity (Templeton et al. 2003a). Heavy metal-resistant bacteria could form a biofilm over sand grains after inoculation. The biofilm was able to efficiently remove Cd, Zn, Cu, Pb, Hg, Ni or Co from wastewater by sorbing or precipitating these heavy metals Nutrients and a carbon source promoted the regrowth of the biofilm on the sand grains (Diels et al. 2003).

Bacteria, attached to metal oxide surfaces, may interfere with sorption by changing the characteristics of the electrical double layer at the solid/solution interface, blocking surface sites, or providing a variety of new sites for metal binding (Templeton et al. 2001, Huang et al. 2005). The sorption and precipitation of Fe^{3+} at the surface of *Shewanella alga* cells were considered to alter the electrochemical surface properties of its composite with iron oxide, buffering the effects of increased ionic strength on subsequent Sr^{2+} sorption (Small et al. 2001).

Small et al. (1999) compared the sorptive capacity of a bacteria-Fe oxide composite with its individual components. *S. alga*, *Shewanella putrefaciens* and the *S. alga*-hydrous ferric oxide (HFO) composite could sorb significant amounts of Sr^{2+} at much lower values of pH (5.5−5.9) compared with HFO by itself (pH 7.6). The sorption capacity of *S. alga*-HFO composite for Sr^{2+} (34 μmol g^{-1}) was less than the combined capacity of its components (41 μmol g^{-1}), indicating the masking of bacterial surface sites by HFO. Similarly, the experimentally measured sorption capacity for Cd^{2+} and Pb^{2+} of the composites of ferrihydrite with *B. subtilis* and *E. coli* was lower than value predicted from adding the available sites (Table 2). The results implied that a masking of reactive bacterial surface sites by ferrihydrite had occurred. Electrophoretic mobility measurements further indicated that the net surface charge of each composite system reflected the surface properties of ferrihydrite rather than those of the bacteria (Kulczycki et al. 2005).

The mobility of heavy metals can change markedly after their sorption by organic or inorganic soil components. Similarly, bacterial cells as such, or as a biofilm over soil particle surfaces, can sequester metals and transform them into less mobile and bioavailable forms. In an attempt to understand the impact of biosorption on metal transport through a mineral system, Yee and Fein (2002) measured the movement of *B. subtilis* and aqueous Cd through quartz and Fe-coated quartz columns as a function of pH. Under some conditions, adsorption of Cd by bacteria and bacterial transport facilitated the migration of Cd through the column. Under other conditions bacterial transport was inhibited and Cd mobility was retarded when the bacteria were sorbed by quartz and/or strained by the sand matrix. Thus, the availability of biosorbed heavy metals in soils may be decreased when the biomass is associated with inorganic soil constituents.

Heavy metals bound to bacteria-soil composites may not be as easily released to the environments as those sorbed by pure bacteria. Flemming et al. (1990) reported that the order of remobilization of heavy metals from bacteria-clay composites was Cr << Ag < Cu. Chromium was very stable when sorbed by bacterial cell walls, clay, and bacterial wall-clay

composites. High Ca concentrations or acidic pH were very effective in mobilizing sorbed metals while organic chelating agents (e.g. EDTA) were less effective. The particle size of the sorbed metal may account for some of the stability changes; those metals that formed large, compact aggregates (Cr and Ag), as seen by transmission electron microscopy, were less likely to be remobilized. Therefore, the remobilization of toxic heavy metals in soils and sediments was considered as a complex process, and predictions based on single inorganic or organic component systems are too simplistic.

Table 2. Sorption capacity (mmol g^{-1}) of bacteria-feerihydrite composites for Cd^{2+} and Pb^{2+}: observed vs. calculated (after Kulczycki et al. 2005)

	Cd^{2+}		Pb^{2+}	
Composite	Observed	Calculated	Observed	Calculated
B. subtilis −ferrihydrite	0.29	0.57	0.5	0.805
E. coli −ferrihydrite	0.15	0.44	0.68	0.775

The remobilization of metals sorbed by bacteria-mineral composites was also influenced by the type of metal and the species of metal ions. Templeton et al. (2003b), for example, showed that a large fraction of the insoluble Se(0) produced within a *B. cepacia* biofilm was retained during exchange with Se-free solutions. They also found that Se (IV) intermediates generated during Se(VI) reduction were preferentially bound to the alumina surface and could not be fully desorbed. On the other hand, Se (VI) was rapidly and extensively remobilized. In a recent study, Huang et al. (2005) reported that Cu^{2+} and Cd^{2+} ions bound by composites of *E. aerogenes* NTG-01 with clay minerals and soil colloids were easily released by NH_4NO_3 and EDTA.

Lebeau et al. (2002) investigated the sorption of cadmium by viable microbial cells that were free or immobilized in alginate beads by incubating the bacteria in a liquid soil extract medium at pH 5−7 and Cd concentrations of 1 to 10 mg L^{-1}. The percentage of Cd biosorbed reached a maximum (69%) at low Cd concentrations and neutral pH. Thus, the effectiveness of bacteria, inoculated into metal-contaminated soils, would largely depend on the concentration of the metal and its distribution between the biomass and the medium.

Conflicting results have been obtained on the ability of free and immobilized bacteria to sorb heavy metals. McEldowney (2000), for example,

found major differences in the ability of *P. fluorescens* H2 to accumulate Cd between the free bacteria (suspended in maleate buffer) and those attached to a glass surface. The time to saturation with Cd^{2+} was different between free and attached cells. Cd^{2+} accumulation by free cells was depressed in the presence of zinc but remained several orders of magnitude higher than that by attached cells. The presence of Zn^{2+} did not inhibit Cd^{2+} uptake by attached cells; instead, uptake increased as the concentration of Cd^{2+} increased. Cd^{2+} accumulation by attached cells increased with pH but decreased by 40% in the presence of a metabolic inhibitor (carbomyl cyanide m-chlorophenyl-hydrazone). These findings suggest that the accumulation of heavy metal by bacterial cells is substantially affected by attachment to solid surfaces. On the other hand, no differences were observed by Lebeau et al. (2002) for the sorption of Cd between free and immobilized cells in a soil extract medium.

Kulczycki et al. (2005) investigated the sorption of Cd^{2+} and Pb^{2+} by ferrihydrite and its composites with *B. subtilis* and *E. coli* at pH 3.0−6.5, and metal concentrations of 1.0×10^{-4} and 3.2×10^{-5} M. The log of the apparent surface complex formation constants ($\log K^{S}_{M}$) and sorption capacity (S_{max}) values were determined by fitting the experimental data to one-site Langmuir sorption isotherms. The one-site model effectively described the sorption data ($r^2 > 0.9$), where Cd^{2+} exhibited lower sorption affinities ($\log K^{S}_{M} = -3$ for ferrihydrite, –1.7 for *B. subtilis*-ferrihydrite, and –1.1 for *E. coli*-ferrihydrite) than Pb^{2+} ($\log K^{S}_{M} = -0.9$ for ferrihydrite, –0.2 for *B. subtilis*-ferrihydrite, and –0.1 for *E. coli*- ferrihydrite). The corresponding S_{max} values for Cd^{2+} and Pb^{2+} were 0.78 and 1.34 mmol g^{-1}, respectively, on ferrihydrite; 0.29 and 0.5 mmol g^{-1}, respectively, on *B. subtilis*-ferrihydrite composites, and. 0.15 and 0.68 mmol g^{-1}, respectively, on *E. coli*-ferrihydrite composites.

The distribution of heavy metals at the bacteria/mineral interface has attracted the attention of several scientists. Templeton et al. (2001) used the long-period X-ray standing wave technique to probe the distribution of aqueous Pb(II) sorbed at the interface between *Burkholderia cepacia* biofilms and hematite (α-Fe_2O_3) or corundum (α-Al_2O_3) surfaces. The formation of a monolayer biofilm on the metal oxide surfaces provided high-energy sites for the sorption of Pb(II) at submicromolar concentrations with uptake decreasing in the order: α-Fe_2O_3 (0001) > α-Al_2O_3 (1102) > α-Al_2O_3 (0001). More recently, Templeton et al. (2003a) measured the partitioning of Pb(II) between *B. cepacia* biofilms and bioflims coated with goethite (α-FeOOH) particles by EXAFS. At least 50% of the total Pb(II) sorbed at pH < 5.5 was associated with the biofilm, while the total uptake (>70% Pb/goethite) at pH > 6 by the composite was dominated by

goethite. Sorption of Pb(II) by the biofilm at high pH was dramatically decreased due to competition with the goethite surface. On the other hand, Pb sorption by goethite was significantly enhanced at low pH compared with systems with no complexing ligands. The bonding mode of Pb(II) in goethite was dependent on [Pb] concentration. Structural fitting of the EXAFS spectra indicated that the Pb-goethite surface complexes, formed at low [Pb] and pH 6, were largely composed of inner-sphere bidentate (binuclear) complexes bridging two adjacent singly coordinated surface oxygens, giving rise to Pb-Fe distances of ~3.9 Å. At high [Pb], however, the dominant complexes on the surface of goethite were bidentate edge-sharing complexes with Pb-Fe distances of ~3.3 Å.

The distribution of Se at the interface of aerobic *B. cepacia* biofilms and α-Al_2O_3 was largely dependent on the speciation of the element (Templeton et al. 2003b). Changes in the partitioning of Se over time are correlated with microbially induced reduction of Se(VI) and Se(IV) to Se(0), as observed by X-ray absorption near edge structure (XANES) spectroscopy. At low [Se], selenite preferentially binds to the alumina surface, while at high [Se] selenite was increasingly partitioned into the biofilm. Metabolically active *B. cepacia* rapidly reduced a fraction of the SeO_3^{2-} to elemental Se(0). Selenate was preferentially partitioned into *B. cepacia* biofilms at all [Se] tested because of its low affinity for the alumina surface. Rapid reduction by *B. cepacia* of SeO_4^{2-} to Se(IV) and then to Se(0) gave rise to a vertical segregation of Se species at the *B. cepacia*/α-Al_2O_3 interface. Elemental Se(0) and Se(VI) accumulated within the biofilm, while Se(IV) intermediates preferentially sorbed to the alumina surface. *B. cepacia*/α-Al_2O_3 samples incubated with SeO_4^{2-} and SeO_3^{2-} when the bacteria were metabolically active caused an appreciable reduction in the mobility of Se as compared with X-ray treated biofilms.

Organic matter is also the essential component of natural soils and its association with microorganisms may influence the behavior and fate of toxic metals. A variety of batch complexation experiments were performed by Borrok et al. (2007) in single, binary and ternary systems for the three components: natural organic matter (NOM), bacterium (*B. subtilis*) and metals (Pb, Cu, Cd, and Ni) to determine the significance of ternary complexation. They found that the formation of bacteria-metal-NOM complex is a rapid, fully-reversible chemical process. The stability of bacteria-metal-NOM complexes increases with the decrease of pH. All NOM fractions form ternary complexes to similar extents at circumneutral pH, but humic acid becomes the dominant NOM fraction in ternary complexes at low pH. The abundance of humic acid in ternary form is greatest with Ni or Cd systems and less with Pb and Cu systems. Their results suggest that

ternary complexes may impact the mobility of aqueous metal cations in natural systems by changing dissolved NOM-metal complexes to colloidal bacteria-metal-NOM complexes which results in decreased bioavailability of the toxic metals.

The formation of bacteria-soil composites may change the surface properties of minerals and their complexes with soil organic matter, and hence alter the behavior of toxic metals at soil particle surfaces. Huang et al. (2000) have attributed the large capacity of red and kaolinite-rich soils for taking up Cd to the abundant presence of rhizobia in these soils. Thus, when they inoculated *Rhizobium fredii* strain HN01 into a Red Soil and a Cinnamon Soil that had been treated with three heavy metals, the amount of Zn associated with carbonate, manganese oxides, and organic matter decreased by 9−26%. Inoculation also depressed the release of Cu to the soil solution, while the total amount of Cu associated with the mineral constituents in the Cinnamon Soil increased. The increase in exchangeable Cu and Cu associated with carbonate, Mn oxides, and organic matter ranged from 20 to 54%. Subsequently, Huang et al. (2004) found that the amount of exchangeable Cd and that of Cd bound to organic matter increased by 22 and 11%, respectively. At the same time, the Cd that was specifically adsorbed and bound to Mn oxides decreased by 14 and 29%, respectively. Inoculation of the Cu- and Cd-tolerant bacterium, *E. aerogenes* NTG-01, into an Alfisol and an Ultisol increased the surface area of the clay fraction in these soils by 3.0–8.8%, and enhanced the sorption of Cd^{2+} and Cu^{2+}. Bacterial inoculation also increased the negative charge, and decreased the positive charge, of the clay fraction over the pH range of 4.0 to 6.5 (Huang et al. 2005).

6 Concluding Remarks

Bacteria and their composites with soil minerals or organic matter are capable of taking up a wide range and variety of toxic metals in soil environments. Research done over the last decade or so has greatly improved our understanding of the mechanisms on biosorption of metals and bacteria-metal-soil component interactions. However, more studies from molecular level are needed in order to enhance the ability of bacteria and their association with soil components to remediate toxic metals-contaminated soils. The focus of future investigations should be on the mechanisms by which metals are sorbed and bound by bacterial cell surfaces and bacteria-soil/mineral composites. In this connection, X-ray absorption spectroscopy (XAS) is a promising technique because it can provide information about

the number and type of near-neighbors for the metals of interest together with estimates of bond distances. Another research imperative is to isolate bacteria from a variety of contaminated soils and associated environments, and elucidate the mechanisms of their tolerance to toxic metals. Molecular biotechnologies, notably DNA recombinant technology for bacterial surface display can yield highly sorptive bacteria (Valls et al. 2000, Deng et al. 2003). The impact of these bacteria on the binding and distribution of toxic metals at the interface of the bacteria-soil composite/solution interface is worth while investigating. Equally important is the association of heavy metal-resistant bacteria with hyperaccumulator plants and its potential in remediating toxic metal-polluted soils.

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4 Adsorption of Biopolymers, with Special Emphasis on Globular Proteins

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1 Introduction

Adsorption of (bio)polymers occurs ubiquitously, and among the biopolymers, proteins are most surface active. Wherever and whenever a protein-containing (aqueous) solution is exposed to a (solid) surface, it results in the spontaneous accumulation of protein molecules at the solid-water interface, thereby altering the characteristics of the sorbent surface and, in most cases, of the protein molecules as well (Malmsten 2003). Therefore, the interaction between proteins and interfaces attracts attention from a wide variety of disciplines, ranging from environmental sciences to food processing and medical sciences.

Proteins are biopolymers of some 22 different amino acids. Because of the variation in physical-chemical properties, mainly polarity and electrical charge, between the constituent amino acids, protein molecules are ampholytic (i.e., containing positively and negatively charged groups) and more or less amphiphilic (i.e. comprising polar and apolar domains). These properties, in turn, lead to the formation of complex three-dimensional (3D) structures.

Soil systems are highly heterogeneous, containing particles of colloidal dimensions. Hence, soil represents a relatively large interfacial area per unit volume, and a large fraction of the surface-active components, e.g., proteins, present in soil are adsorbed at interfaces. This has a number of consequences. For instance, by being adsorbed at surfaces, the hydrolysis of proteins by proteases (from micro-organisms) may be affected and, therefore, their availability as a nutrient. Further, the structure of a protein molecule and, hence, its biological activity are influenced by changes in its environment (Haynes and Norde 1994; Norde et al. 2005), as occurring during adsorption. This would affect the biological functioning of extracellular enzymes. Also, the colloidal stability of soil particles is strongly influenced by electrosteric effects caused by adsorbed protein molecules. Colloidal stability of soil systems is of prime importance in soil structure.

Whatever the mechanism of the adsorption process, it occurs spontaneously, at constant temperature and pressure, only if the Gibbs energy, G, of the system decreases:

$$\Delta_{\text{ads}} G = \Delta_{\text{ads}} H - T\Delta_{\text{ads}} S < 0 \tag{1}$$

where H, S, and T refer to the enthalpy, entropy, and temperature (in degrees Kelvin) and Δ_{ads} indicates the change resulting from the adsorption process.

The tendency of proteins to adsorb at interfaces is determined by many variables, including the pH, the ionic strength, the properties of the protein molecules and the interfaces, and the nature of the solvent and other components present. The process of protein adsorption is complicated, and despite the great volume of work over the past decades, a unified theory is still far ahead. Yet, some principles may be indicated.

Discussing principles of protein adsorption may start from general trends observed for the adsorption of more simple flexible, highly solvated polymers, in particular, polyelectrolytes.

2 Flexible Polymers

Flexible, coily-structured polymers in solution possess a high conformational entropy resulting from the many rotational possibilities of the single bonds in the polymer chain.

The expansion of a polymer coil is determined by its interaction with the solvent. The more favorable the interaction between the polymer segments and the solvent molecules (good solvent), the better the polymer dissolves and the more the coil expands.

Adsorption of the polymer molecule causes a reduction of its conformational entropy (Norde 2003b). Hence, adsorption takes place only if the loss in conformational entropy is compensated by sufficient favorable interactions between polymer segments and the interface. Because the polymer molecule attaches with many segments at the interface, it adsorbs tenaciously with a very high affinity, even if the interaction of the individual segments with the interface is rather weak. The high affinity manifests itself by the adsorption being irreversible with respect to variations of the polymer concentration in solution.

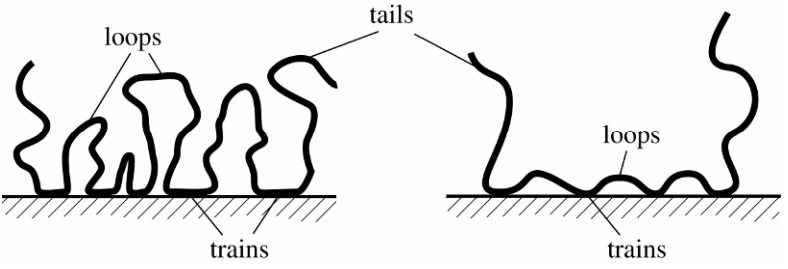

Fig. 1. Conformations of a flexible polymer molecule adsorbed from a poor solvent (*left*) and from a good solvent (*right*).

Figure 1 illustrates how the segments of an adsorbed flexible polymer molecule may be distributed among trains, loops, and tails. Trains refer to the attached segments. They are rarely long, and they do not completely occupy the surface, leaving about 20–50% of the surface uncovered. As a rule, loops account for most of the adsorbed mass, their extension is primarily determined by the solvent quality. A high loop density is tolerated only if the solvent quality is poor (relatively unfavorable polymer-solvent interaction). Then, the maximum amount of polymer that can be accommodated in an adsorbed layer is in the range of, approximately, 2–5 mg m^{-2}. For a good solvent, the adsorbed polymer layer is more dilute and less

thick, reaching a value of, typically, 0.5–2 mg m^{-2}. For entropic reasons, tails usually extend far into the solvent. The high affinity character of polymer adsorption is reflected in the shape of the adsorption isotherm, where the adsorbed mass, Γ, per unit area of the sorbent surface, is plotted against the polymer concentration, c_p, in solution. Typical isotherms for polymer adsorption are shown in Fig. 2. The initial part of the isotherms practically merges with the Γ-axis, because at low polymer supply, all of the polymer adsorbs until the surface is saturated and the plateau value of the isotherm is reached.

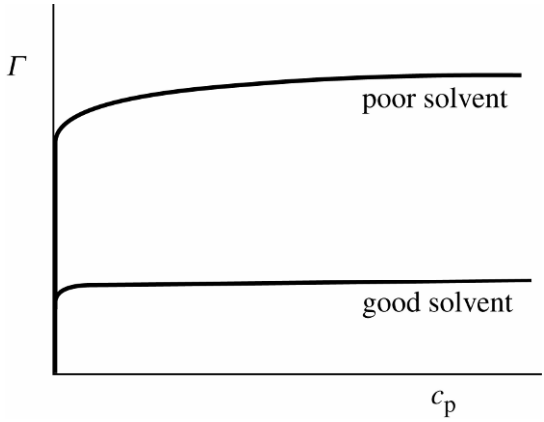

Fig. 2. Schematics of adsorption isotherms for polymers, where the adsorbed mass Γ per unit area of the sorbent surface is plotted against the polymer concentration c_p in solution. The steep initial slope of the isotherms indicates high adsorption affinity, which is typical for polymers. The plateau-value of Γ is strongly determined by the conformation the adsorbed polymer molecules adopt (see Fig. 1), which, in turn, is strongly influenced by their solubility in the solvent.

The same considerations apply to the adsorption behavior of polyelectrolytes, i.e., polymers that carry electrically charged groups (anionic or cationic) along their chain. Because of the intramolecular repulsion between like-charged groups, polyelectrolytes are strongly expanded in aqueous solution, and water is a good solvent for flexible polyelectrolytes. Consequently, the formation of thick dense loops in the adsorbed layer is strongly suppressed, and polyelectrolytes adsorb in amounts of less than a few mg m^{-2}. As with uncharged polymers, to adsorb flexible polyelectrolytes requires a critical attractive interaction with the

sorbent surface to compensate for the loss of conformational entropy. In addition to a chemical, nonelectrostatic interaction, polyelectrolytes interact electrostatically with the sorbent surface, provided that the surface is also electrically charged (which is usually the case in an aqueous environment). Depending on the charge signs of the polyelectrolyte and the sorbent surface, the electrostatic interaction is attractive or repulsive. It may or may not outweigh the nonelectrostatic interaction. For example, the electric contribution to $\Delta_{ads}G$ from a monovalent ionic group in an electric field is about 1 RT (R being the universal gas constant, and T the temperature in degrees Kelvin) for every 25 mV, and the contribution from dehydration of a $-CH_2-$ group is about 1.1 RT (Tanford 1973), both at room temperature. Thus, polyelectrolytes with some hydrocarbon groups in their chain may readily adsorb under electrostatically unfavorable conditions.

In contrast to uncharged polymers, the adsorption of polyelectrolytes is highly sensitive to variations in ionic strength. At elevated ionic strength, charge-charge interactions may be effectively screened and the polyelectrolyte behavior approaches that of an uncharged polymer. This effect is reflected in the shape of the adsorption isotherms, as indicated in Fig. 3a and 3b. The pH, which usually controls the charge on the polyelectrolyte and, often, on the sorbent surface, influences adsorption in a similar way: at a pH where the polyelectrolyte is only weakly charged, it adsorbs in a thicker loopy layer with less sensitivity to variation in ionic strength.

Following similar reasoning, the adsorption pattern observed for ampholytic polyelectrolytes 0can be explained. As illustrated in Fig. 4, polyampholytes show maximum adsorption around their isoelectric point (i.e., the pH where the net charge of the polyampholyte is zero).

Biopolymers e.g., polysaccharides, polynucleotides, unfolded protein molecules, that all attain expanded flexible structures in solution adsorb more or less according to the principles discussed above.

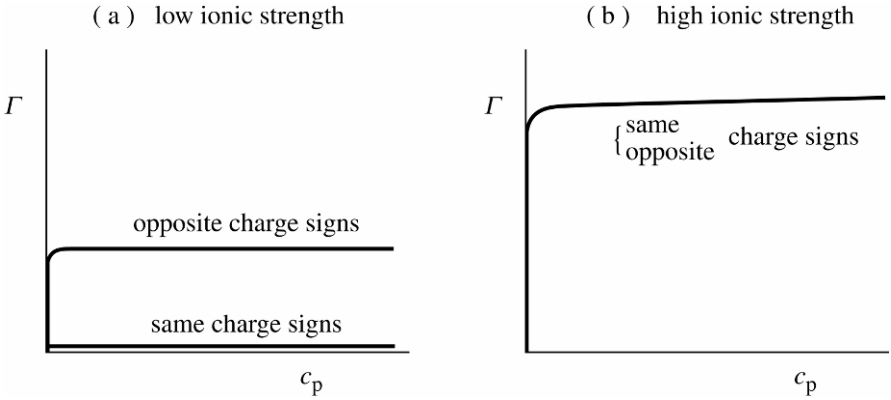

Fig. 3. Schematics of the influence of electrostatic interactions on adsorption isotherms of polyelectrolytes. Effect of charge contrast between the polyelectrolyte and the sorbent surface in media of (**a**) low and (**b**) high ionic strength.

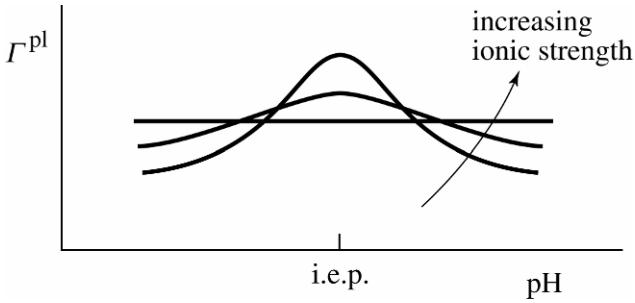

Fig. 4. Influence of pH on the plateau-value Γ^{pl} of adsorption isotherms of polyampholytes. At either side of the isoelectric point, i.e.p., the polyampholyte attains a net charge causing intra- and intermolecular electrostatic repulsion. As a result, the mass of adsorbed polyampholyte, that can be accommodated per unit area of the sorbent surface, decreases. Electrostatic interactions are suppressed by increasing ionic strength, yielding Γ^{pl} less sensitive to pH.

3 Globular Proteins

The structure and structural stability of globular proteins in aqueous solution are the result of various interactions inside the protein molecule, between the protein and the water, and among the water molecules (Norde 2003a). The

sequence of the amino acids in the polypeptide chain ultimately determines the spatial architecture, i.e., the 3D structure that a protein molecule adopts.

In contrast to flexible polymers, the 3D structures of globular proteins are usually highly organized and contain different well-defined structural elements, such as α-helices and β-sheets (Creighton 1993). Thus, globular proteins represent low-conformational entropy states of the polypeptide chain. Because the various types of interactions that are involved in shaping the 3D structure of a protein counteract each other, the resulting (native) structure is only marginally stable. However, the adsorption of globular proteins shares some features with that of flexible polymers, but in various aspects it is different. Similar to flexible polymers, proteins adsorb via multiple contacts, which, more often than not, results in high affinity adsorption. Furthermore, the effects of pH (i.e., electric charge of the protein) and ionic strength follow the general pattern observed for a polyampholyte: the adsorbed mass is often found to be maximal at the isoelectric point of the protein, and the pH dependency reduces with increasing ionic strength (Haynes and Norde 1994). Despite their internal coherence, adsorbed globular proteins also tend to spread over the surface to optimize its interaction with the surface. However, proteins retain more or less their compact structure; in other words, the polypeptide chain does not unfold into a loop-and-train like structure as observed for flexible polymers.

A change in the environment of a protein molecule, e.g. adsorption from aqueous solution onto a sorbent surface, may lead to a partial breakdown of its ordered structure, resulting in an increase of conformational entropy. This is a fundamental difference between protein adsorption and the adsorption of flexible polymers, for which attachment to a surface implies a loss of conformational entropy.

The main contributions to $\Delta_{\mathrm{ads}}G$ are discussed in Sect. 3.1

3.1 Types of Interaction Involved in Protein Adsorption at a Smooth Surface

The main contributions to $\Delta_{\mathrm{ads}}G$ for a globular protein are from electrostatic, dispersion, and hydrophobic forces and from changes in the structure of the protein molecule. Although in this section these contributions are discussed individually, strict separation of the influence of these forces on the overall adsorption process of a protein is not possible. For instance, adsorption-induced alteration of the protein structure affects the electrostatic and hydrophobic interaction between the protein and the surface. When the sorbent surface is not smooth but is covered with (polymeric)

protuberances ('hairy' surfaces), additional, mainly steric, interactions come into play. Hairy surfaces are often encountered in nature as a result of adsorbed or grafted natural polymers, such as polysaccharides, that reach out in the surrounding medium with some flexibility. Interaction of proteins with such hairy surfaces will be discussed in Sect. 3.3.

3.1.1 Interaction Between Electrical Double Layers

As depicted in Fig. 5, both the protein molecule and the sorbent surface are electrically charged. In an aqueous environment, they are surrounded by counterions, which, together with the surface charge, form the so-called electrical double layer. The Gibbs energy of an electrical double layer, G_{cd}, may be calculated as the isothermal, isobaric reversible work required to invoke the charge distribution in the double layer

$$G_{cd} = \int_0^{\sigma_0} \psi_0' \, d\sigma_0' \tag{2}$$

where ψ_0' and σ_0' are the variable surface potential and surface charge density, respectively, during the charging process. Integration of (2) requires $\psi_0'(\sigma_0')$, and this functionality can be derived from a model for the electrical double layer. To calculate $\Delta_{ads}G_{cd}$, (2) has to be applied three times, i.e., G_{cd} for the bare sorbent surface and for the dissolved protein molecule has to be subtracted from $\Delta_{ads}G_{cd}$ for the protein-covered surface. Charge distributions for the system before and after adsorption are schematically depicted in Fig. 5. For a bare sorbent surface, the Gouy-Stern model (Lyklema 1995) for the electrical double layer may be taken. For a dissolved protein, a discrete charge model (e.g. Kirkwood's model (Kirkwood 1934)) seems to be more appropriate, and for the protein-covered surface charge, distribution models have been proposed by Norde and Lyklema (1978c) and by Ståhlberg et al. (1995). As mentioned before, although usually structurally perturbed, the adsorbed protein molecules retain a compact conformation, and the adsorbed layer usually reaches a thickness of a few to a few tens of nm. Under most ambient conditions of ionic strength, the distance over which electrostatic forces are effective, the Debye length (Lyklema 1991b), is less than the thickness of the adsorbed layer. For instance, in a medium of 0.01 M ionic strength, the Debye length is 3 nm and in 0.1 M it is only 1 nm. Therefore, the compact protein layer shields the contact region between the protein and the sorbent

from electrostatic interaction with the solution. To prevent an excessively high electric potential, the charge density in the nonaqueous contact region (which has a low dielectric constant) must be regulated to be essentially zero. Charge regulation may occur through changes in the ionization of the protein and/or the sorbent surface (Norde and Lyklema 1978a; Haynes et al. 1994) and also by the incorporation of the ions other than charge-determining ions (so-called indifferent ions) (Norde and Lyklema 1978b; Van Dulm et al. 1981). As a result, $\Delta_{ads}G_{cd}$ is not very sensitive to the charge on the protein and the sorbent surface before adsorption, and it usually does not exceed a few tens of RT per mole of protein (Norde and Lyklema 1979). In addition to contributing to the charge regulation, ion transfer between the solution and the adsorbed layer includes a change in the chemical environment of the ions. Compared to water, the low-dielectric proteinaceous environment is a poorer 'solvent' for most ions, and, hence, the chemical effect of incorporation of ions in the adsorbed layer opposes protein adsorption. This explains why protein adsorption often reaches its maximum affinity when the charge densities on the dissolved protein and the bare sorbent surface just match each other, so that no additional ions have to be incorporated to neutralize the protein-sorbent region.

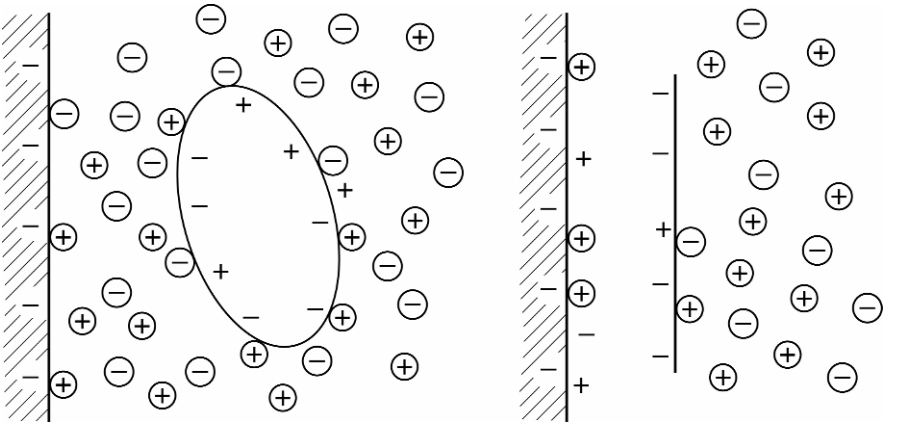

Fig. 5. Schematic representation of charge distributions before (*left*) and after (*right*) protein adsorption. The charge on the sorbent surface and the protein molecule are indicated by $+/-$. The low molecular weight ions are indicated by $\oplus$ and $\ominus$.

3.1.2 Dispersion Interaction

Dispersion interaction arises from the synchronization of the motions of electrons in electron 'clouds' that are in close proximity, thereby inducing

dipoles. For a sphere 1 (e.g., the protein molecule), separated from a body 2, having a planar surface (e.g., the sorbent surface) across a medium 3, the contribution from dispersion interaction to the Gibbs energy of adsorption may be approximated by (Lyklema 1991a)

$$\Delta_{\text{ads}}G_{\text{disp}} = -\frac{A_{12(3)}}{6}\left[\frac{a}{h} + \frac{a}{h+2a} + \ln\frac{h}{h+2a}\right] \tag{3}$$

where $A_{12(3)}$ is the Hamaker constant for the system, a is the radius of the sphere, and h is the separation distance between the sphere and the planar surface.

For $h << a$ (3) reduces to

$$\Delta_{\text{ads}}G_{\text{disp}} = -\frac{A_{12(3)}a}{6h} \tag{4}$$

The Hamaker constant, $A_{12(3)}$, for the system may be derived from those of the individual components, according to

$$A_{12(3)} = \left(A_{11}^{1/2} - A_{33}^{1/2}\right)\left(A_{22}^{1/2} - A_{33}^{1/2}\right) \tag{5}$$

In aqueous media, usually $A_{11} > A_{33}$ and $A_{22} > A_{33}$ and, hence, $A_{12(3)} > 0$, so that $A_{\text{ads}}G_{\text{disp}} < 0$, which implies attraction between 1 and 2. According to (3) and (4), that attraction increases with increasing size of the sphere, and it drops off steeply with increasing separation distance. Further,

$$A_{12(3)} = \left(A_{131}A_{232}\right)^{1/2} \tag{6}$$

The Hamaker constant for interaction across water is about 6.5×10^{-21} J for globular proteins (Nir 1977) and $2-5\times10^{-20}$ J for such oxides as silica and metal oxides (Lyklema 1991c). Based on these values and applying (6) and (4) to a spherical protein molecule having a radius of 3 nm at a distance of 0.1 nm from the surface of a soil particle, $\Delta_{\text{ads}}G_{\text{disp}}$ at 20ºC amounts to $10-20$ RT per mole of protein. Because of the various approximations involved, these values are only semi-quantitative. More

accurate estimates of $\Delta_{ads}G_{disp}$ require more detailed knowledge of the system's parameters, i.e., the Hamaker constants and sizes and shapes of the protein molecules and the soil particles.

3.1.3 Changes in the State of Hydration

Polar groups interact favorably with water molecules, mainly through hydrogen bonding. They compete successfully with hydrogen bonds between the water molecules, making them readily soluble in water. Apolar groups do not offer the possibility of such favorable interactions with water, and they are, therefore, expelled from an aqueous environment. The water molecules in contact with a surface of apolar material are strongly oriented so as to form as many hydrogen bonds as possible to other water molecules, as they can not be formed with the apolar surface. As a result, the entropy of the water adjacent to the surface is greatly reduced. This is the hydrophobic effect.

Protein molecules contain both polar and apolar groups. For proteins dissolved in water, these apolar groups tend to be buried in the interior of the globular structure, as a result of expulsion by the surrounding water. However, other interactions, as well as geometrical constraints, interfere with the hydrophobic effect, so that a minor fraction of the water-accessible surface of the protein molecule may be apolar. Protein molecules that do not spontaneously aggregate in water do not have pronounced apolar patches at their surfaces.

The surfaces of sorbent materials, e.g., oxide particles in soil, are often less complex than the exterior of protein molecules. However, if such particles are (partly) covered with organic materials, e.g., humic acids and/or fulvic acids, their surface chemistry may be very complex as well. Also, surfaces of biological structures, such as those of plant roots, may be heterogeneous.

When the surfaces of the protein molecules and the sorbent are predominantly polar, it is probable that some hydration water is retained between the adsorbed protein layer and the sorbent surface. Then, the contribution from changes in the state of hydration to the Gibbs energy of protein adsorption, $\Delta_{ads}G_{hydr}$, will be minor. When the surfaces are apolar, dehydration is a strong driving force for adsorption. The value of $\Delta_{ads}G_{hydr}$ for apolar surfaces may be approached from partitioning model compounds between water and a nonaqueous solvent (Némethy and Scheraga 1962). It is, thus, estimated that dehydration of an apolar surface lowers the Gibbs energy by about 10–20 mJ m^{-2} (Richards 1977). For a

protein of 30,000 Da and an adsorbed mass of 1 mg m^{-2}, it corresponds, at ambient temperature, to $\Delta_{ads}G_{hydr}$ ranging between –120 *RT* and –240 *RT* per mole of protein. It demonstrates that the contribution from apolar dehydration dominates over those from electrical double layer overlap and dispersion interaction.

3.1.4 Rearrangements in the Protein Structure

The 3D structure of a native protein (in aqueous solution) is only marginally thermodynamically stable and it is sensitive to changes in its environment. It is, therefore, not surprising that adsorption is often accompanied by rearrangements in the protein's 3D structure. It is commonly observed experimentally that the thickness of an adsorbed protein layer is comparable to the dimensions of the protein molecule in solution. It indicates that the adsorbed protein molecules remain compactly structured.

After adsorption, one side of the protein molecule is oriented towards the sorbent surface and turned away from the aqueous solution. As a consequence, apolar parts of the protein that are buried in the interior of the dissolved molecule may become exposed to the sorbent surface, where they are still shielded from contact with water. Because hydrophobic interaction between apolar amino-acid residues in the protein's interior support the formation of such secondary structures as α-helices and β-sheets (Creighton 1993), a reduction of this interaction destabilizes such structures. Breakdown of the α-helices and/or β-sheets content is, indeed, expected to occur if peptide units released from these ordered structures can form hydrogen bonds with the sorbent surface. This is the case for oxides, e.g., silica and metal oxides, which may be abundantly present in soil systems, and with sorbents retaining residual water at their surfaces. Then, the decrease in ordered secondary structures leads to an increased conformational entropy of the protein. This may favor the protein adsorption process by tens of *RT* per mole of protein (Zoungrana et al. 1997; Norde and Favier 1992; Kondo et al. 1992; Kondo et al. 1991). However, if in the nonaqueous protein-sorbent contact region hydrogen bonding between peptide units and the sorbent surface is not possible, as is the case for apolar surfaces, adsorption may induce extra peptide-peptide hydrogen bonds, thereby promoting the formation of α-helices and β-sheets (Zoungrana et al. 1997; Norde and Favier 1992; Kondo et al. 1992, 1991; Norde and Giacomelli 1999). Thus, whether adsorption on an apolar surface results in an increased or decreased order in protein structure depends on the subtle balance between energetically favorable

intra- (and inter-) molecular hydrogen bonding and ensuing changes in the conformational entropy of the protein molecule.

Based on these contributions (a–d), we may arrive at the predictive scheme presented in Table 1. Because of the relatively large contribution from dehydration, essentially all proteins adsorb from an aqueous environment on apolar surfaces, even under electrostatically adverse conditions. With respect to polar surfaces, distinction may be made between proteins having a strong internal coherence ('hard' proteins) and those having a weak internal coherence ('soft' proteins). The hard proteins adsorb at polar surfaces only if they are electrically attracted, whereas the structural rearrangements (i.e., reductions in ordered structure) in the soft proteins lead to a sufficiently large increase in conformational entropy to make them adsorb at a polar, electrostatically repelling surface.

Table 1. Predictive scheme of protein adsorption. The '+' and '–' indicate the electrical charge sign on the sorbent surface and the protein molecule. Conditions at which adsorption is predicted is marked 'yes' and predictions of absence of adsorption is marked 'no'. Further explanation is given in the text

			Sorbent surface			
			Hydrophobic		Hydrophilic	
			+	–	+	–
Protein	Hard	+	yes	yes	no	yes
		–	yes	yes	no	no
	Soft	+	yes	yes	yes	yes
		–	yes	yes	yes	yes
			hydrophobic dehydration dominates adsorption		structural changes in proteins dominate adsorption	

3.2 Protein Adsorption in Model Systems

The model systems, discussed here, contain one type of well-defined protein and one type of well-characterized solid surface in an aqueous medium containing one type of low molecular-weight electrolyte. Table 2 summarizes some relevant properties of the proteins. Lysozyme (LSZ)

from hen's egg, ribonuclease (RNase) from bovine pancreas, and α-lactalbumin (αLA) from bovine milk are relatively small proteins, and they have nearly the same sizes and shapes. They have different isoelectric points, so that for a given pH, these proteins have different net charges. The values of the denaturation temperature and of the Gibbs energy of denaturation indicate that the stability of the native structure in solution decreases in the order LSZ > RNase > αLA. The structural stability of αLA further decreases by removing the Ca^{2+}-ion from the protein. Bovine serum albumin (BSA) is about five times larger than the other proteins. The isoelectric point is at pH 4.6, and its structural stability is comparable to that of αLA.

Table 2. Some properties of proteins relevant for their adsorption behaviour

Protein	LSZ	RNase	αLA	αLA($-Ca^{2+}$)	BSA
Molar mass (Da)	14600	13680	14200	14200	67000
Isoelectric point (pH units)	11.1	9.4	4.3	4.1	4.6
Denaturation temperature (ºC) (at pH of maximum stability)	76	70	63	41	65
Gibbs energy of denaturation ($J\ g^{-1}$)					
heat	4.1	3.2	1.5	–	–
denaturant	4.0	3.9	1.9	0.7	–

LSZ: lysozyme; RNase: ribonuclease; αLA: α-lactalbumin: αLA($-Ca^{2+}$): Ca^{2+} depleted αLA; BSA: bovine serum albumin.

Table 3. Some properties of the sorbent particles

	Electrolyte (0.05 M)				
	Phosphate buffer pH 7.0			Acetate buffer pH 5.5	Borate buffer pH 9.5
	PS^+	PS^-	SiO_2^-	$\alpha Fe_2O_3^+$	$\alpha Fe_2O_3^-$
Nature of charged groups	$=^+NH-$	$-OSO_3^-$	$-O^-$	$-OH_2^+$	$-O^-$
Charge density (mC m^{-2})	+27	–23	–	–	–
Zeta potential (mV)	+32	–69	–39	+20	–47
Hydrophobicity (contact angle with water)	82°	82°	0°	hydrophilic	
Surface area (m^2 g^{-1})	12.4	10.0	100	36.0	36.0

PS: polystyrene; SiO_2: silica; αFe_2O_3: hematite. The superscripts '+' and '-' indicate the sign of the charge on the sorbent particles.

The sorbent materials are supplied as finely dispersed colloidal particles, whose surfaces are smooth. Some of their properties are presented in Table 3. The sorbents cover different combinations of hydrophobicity and sign of the surface charge. Thus, the model systems presented allow systematic investigation of the influences of hydrophobicity, electric charge, and protein structural stability on protein adsorption.

The protein adsorption isotherms (not shown here) for all these systems show well-developed plateau values, Γ^{pl}. Values of Γ^{pl} are given in Fig. 6. The charge of the proteins is qualitatively indicated by '+' and '–' signs. At the hydrophobic surfaces of polystyrene (PS), all proteins adsorb, both under electrostatically attractive and repulsive conditions. At the hydrophilic surface of hematite (αFe_2O_3), where dehydration does not favor adsorption, the structurally most stable proteins, LSZ and RNase, adsorb only if electrostatically attracted, but the less stable proteins, αLA and BSA, adsorb even when electrostatically repelled. Adsorption-induced loss of ordered secondary structure in these proteins has been inferred from circular dichroism experiments (Norde and Giacomelli 1999). The resulting increase in conformational entropy of the polypeptide chain is

apparently sufficiently large to cause spontaneous adsorption of these proteins at a hydrophilic, electrostatically-repelling surface. A similar behavior is observed at the hydrophilic surface of silica (SiO_2); here, the influence of the structural stability of the protein is further demonstrated by the differences between the Γ^{pl}-values of αLA and αLA($-Ca^{2+}$).

The experimental data presented in Fig. 6 are in accordance with the predictions given in the scheme of Table 1.

3.3 Morphology of the Sorbent Surface

It is assumed in the foregoing that the surface at which the biopolymer adsorbs is smooth. However, in quite a few cases, sorbent surfaces may be 'hairy'. For instance, at biological surfaces (e.g., those of biological membranes and microbial cells) natural polymers, such as polysaccharides, are often present. Soil particles may also be (partly) covered with polymeric substances, e.g., humic and fulvic acids. When these polymers 'reach out' with a relatively high degree of flexibility in the surrounding medium (cf., Sect. 2), the surface will dynamically respond to incoming globular protein molecules. It may offer the possibility of optimizing contact by conforming to the shape of the adsorbing protein molecule. However, when the density of the extending polymer segments (loops and tails) of the flexible polymer at the surface is high, the small separation distance between these segments ('hairs') may hamper the ability of the protein molecule to penetrate the polymer layer. An incoming protein molecule then squeezes the polymer hairs, causing a locally higher polymer concentration. This results in an increased osmotic pressure in the polymer layer, which gives rise to repulsion of the incoming protein molecule. As a result, protein adsorption is suppressed (Halperin 1999; Szleifer 1997). In model systems where polyethylene oxide (PEO) is used, polymer layers have proven to be successful in controlling protein adsorption (Norde et al. 2005; Currie et al. 2003). The effect of preadsorbed or end-grafted polymers on protein adsorption depends primarily on two characteristics of the polymer layer:

(i) the grafting density of the polymer chains (hairs).

(ii) the extension of the polymer chains in the surrounding solution.

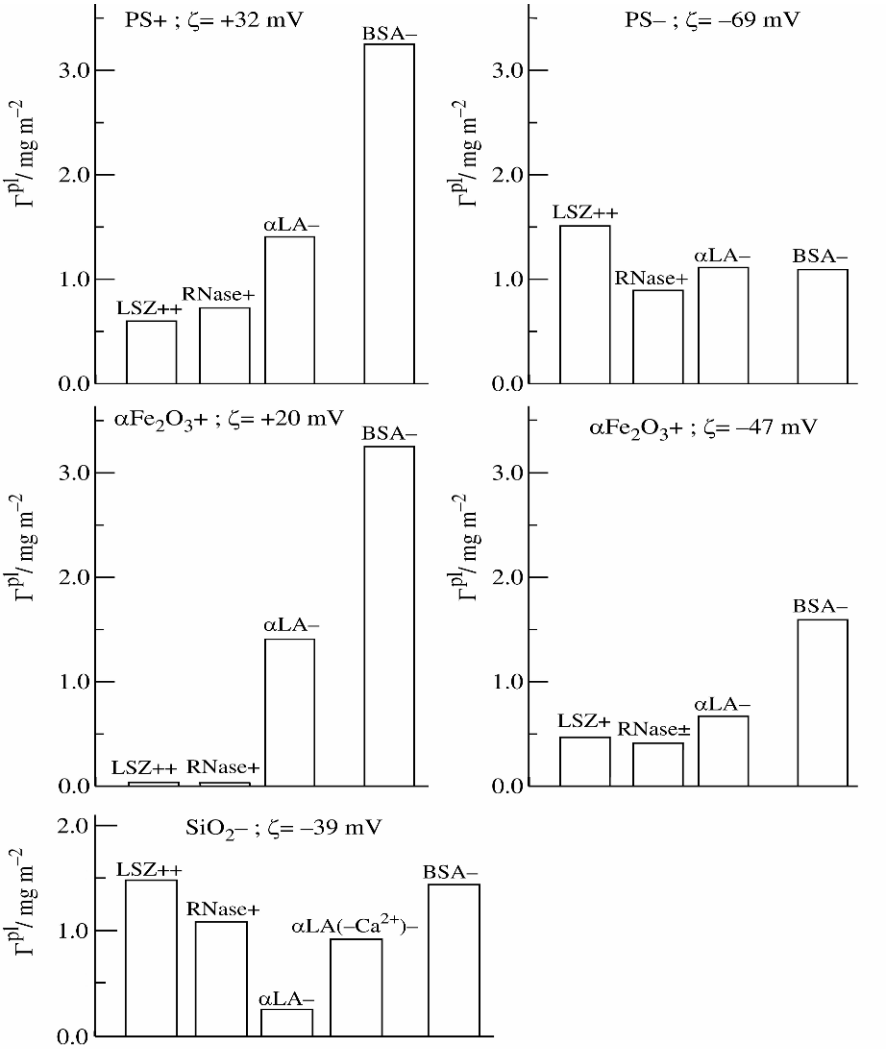

Fig. 6. Plateau-values, $\Gamma^{pl}/\mathrm{mg\,m^{-2}}$, of adsorption isotherms of lysozyme (LSZ), ribonuclease (RNase), α-lactalbumin (αLA), calcium-depleted α-lactalbumin ($\alpha LA(-Ca^{2+})$) and bovine serum albumin (BSA) on hydrophobic polystyrene (PS) and hydrophilic hematite ($\alpha - Fe_2O_3$) and silica (SiO_2) surfaces. An indication of the charge density of the surface is given by the zeta-potential, ζ, and of the proteins by '+' and '–' signs. Ionic strength 0.05 M; T = 25ºC. (Derived from Currie et al. 2003).

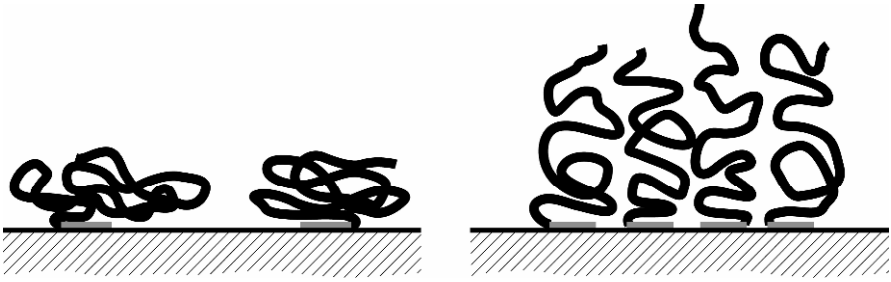

Fig. 7. Conformational states of polymer chains grafted at a surface. Influence of grafting density: mushroom (*left*) and brush (*right*).

Despite controversy in the literature, some trends emerge. As expected, protein adsorption is reduced with increasing grafting density of the polymer. When the separation distance between the polymer chains becomes smaller, the chains have to stretch out into the solution. The polymer layer is then said to attain a brush conformation (Fig. 7). The conformation of the brush determines the efficacy of protein repulsion. A higher brush density is required to suppress the adsorption of smaller protein molecules. As a rule, protein repellency increases with increasing length of the polymer chains, although this effect may be more complex, as illustrated by the results of Currie et al. (1999) (Fig. 8), which show that at relatively low grafting densities, long PEO chains in a brush enhance protein adsorption. Similar results were reported by Norde and Gage (2004). It implies that in addition to steric and osmotic repulsion forces (weak) attractive interaction between the polymer and the protein exists (Efremova et al. 2001; Sheth et al. 2000). Furthermore, in the case of short polymer chains forming a brush of low thickness, long-range dispersion and/or electrostatic forces may cause accumulation of protein molecules at the outer edge of the brush. In such a mode of adsorption, an intimate contact of the protein molecules and the sorbent surface is prevented. Consequently, the adsorbed protein molecules are expected to be less structurally perturbed and, hence, to retain biological activity. An example is given in Sect. 3.4 before.

3.4 Adsorption-Induced Changes in the Structure and Biological Activity of Proteins. A Case Study

Because of the structure-function relationship for (globular) proteins, adsorption-induced changes in the molecular structure are likely to affect the biological activity of the protein, e.g., the enzymatic activity. In soils, as well as in a wide variety of other systems, the impact on biological

functioning is the most relevant aspect of adsorption. Here, the following case study is briefly discussed as a typical example.

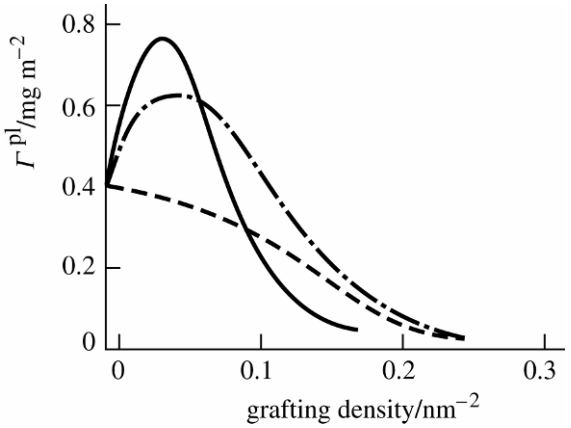

Fig. 8. Adsorption of BSA on surfaces coated with polyethylene oxide (PEO). Influence of the grafting density of PEO at the surface for different polymer chain lengths (—— 700, −·−·− 445, and − − − 148 ethylene oxide monomers) on the plateau-adsorption, Γ^{pl} / mg m^{-2} of BSA. (Redrawn from Sheth et al. 2000).

Zoungrana et al. (1997) and Norde and Zoungrana (1998) investigated the influence of adsorption on the structure, structure stability and biological activity of a proteolytic enzyme, α-chymotrypsin. The enzyme was adsorbed from 0.01 M phosphate buffer at pH 7.0 and at 22ºC onto solid surfaces of different hydrophobicities and morphologies.

The sorbents were hydrophobic Teflon, hydrophobic polystyrene (PS), and hydrophilic silica. These sorbents were negatively charged colloidal particles having smooth surfaces. In adition, PS particles at the surface of which oligomers (8-mers) of ethylene oxide ($(EO)_8$) were grafted at a density of one $(EO)_8$-moiety per 2.5 nm^2 were used. Because of the water-solubility of EO, these flexible $(EO)_8$ oligomers reach out from the surface into the aqueous solution, causing a hairy sorbent surface. For a more detailed description of these sorbent materials, see Zoungrana and Norde (1997), and Norde and Zoungrana (1998).

The molecular shape of α-chymotrypsin is an ellipsoid of 5.1 nm × 4.0 nm × 4.0 nm. Its molar mass is 25,200 Da and its isoelectric point is 8.1.

Figure 9 shows adsorption isotherms for this protein on the various sorbents. As is usually observed for proteins at surfaces, the adsorption affinity is higher for the hydrophobic surfaces than for the hydrophilic ones. The adsorption plateau-values at silica and PS-$(EO)_8$, approximately 2.5 mg m^{-2}, are compatible with a complete monolayer of side-on adsorbed molecules of α-chymotrypsin. Adsorption saturation on the PS and, even more so, the Teflon surfaces is beyond monolayer coverage, suggesting that on these hydrophobic surfaces the protein molecules are so severely perturbed as to accommodate more protein mass in the adsorbed layers and/or there is adsorption of a second layer of protein molecules (possibly triggered by structurally altered molecules in the first layer). Modifying the PS-surface with $(EO)_8$ moieties lowered the adsorption affinity. In view of the dimensions of the α-chymotrypsin molecules and of the $(EO)_8$ grafting density, it is inferred that the surface area per adsorbed α-chymotrypsin molecule comprises about 8 $(EO)_8$-oligomers. This would prevent intimate contact between the adsorbed protein molecules and the PS surface. As a result, the structural integrity of the protein is expected to be less perturbed.

The influence of adsorption on the structure of α-chymotrypsin is shown in Fig. 10, where the circular dichroism (CD) spectrum of the protein in solution is compared with that of the protein adsorbed on Teflon and silica. Because of absorbance in the far UV by the aromatic styrene, it is impossible to obtain reliable CD spectra of proteins adsorbed on PS and PS-$(EO)_8$. The CD spectrum of a protein reflects its composition of secondary structural elements (α-helices, β-sheets). The spectrum of dissolved α-chymotrypsin is indicative of a low content of α-helices and a high content of β-sheets. After adsorption at the silica surface, the CD spectrum is shifted, but the shift is much more pronounced when the protein was adsorbed at the Teflon surface. The shifts are in opposite directions for the hydrophobic and hydrophilic surfaces, respectively. The spectrum of the protein on the hydrophilic surface of silica indicates a decrease in ordered secondary structure, i.e., the polypeptide chain in the protein has an increased random structure and, hence, a larger conformational entropy. Adsorption on the hydrophobic Teflon surface induces the formation of ordered structural elements, notably an increase in the content of α-helices (cf., the discussion in Sect. 3.1.4).

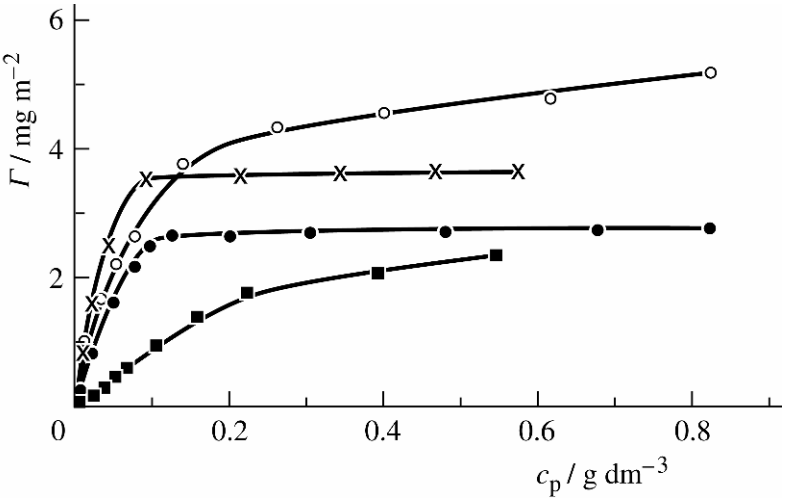

Fig. 9. Adsorption isotherms where the adsorbed mass $\Gamma / \mathrm{mg\ m^{-2}}$, of α-chymotrypsin on various sorbent surfaces is plotted against the protein concentration $c_p / \mathrm{g\ dm^{-3}}$, in solution. Sorbents: Teflon (○), polystyrene (x), polystyrene $-(\mathrm{EO})_8$ (●), silica (■). Conditions: 0.01 M phosphate buffer pH 7.1; 22ºC. (Redrawn from Zoungrana and Norde 1997).

The enzymatic activities of α-chymotrypsin in solution and adsorbed at the different surfaces are presented in Fig. 11, where the specific enzymatic activity (defined as activity per unit mass of protein) is plotted as a function of temperature. The enzyme loses activity due to adsorption. On the hydrophobic Teflon and PS surfaces, the activity is completely gone, whereas on the hydrophilic silica surface, α-chymotrypsin has retained most of its biological function. These differences are in agreement with the adsorption isotherms and the circular dichroism spectra. The influence of the hydrophobicity of the sorbent surface on the affinity of the protein for the sorbent surface, as judged from the rising parts of the adsorption isotherms (Fig. 8), suggests that the proteins are more perturbed and, hence, less biologically active when adsorbed at hydrophobic surfaces. Also, the CD spectra indicate that adsorption-induced structural perturbations are more severe at hydrophobic surfaces.

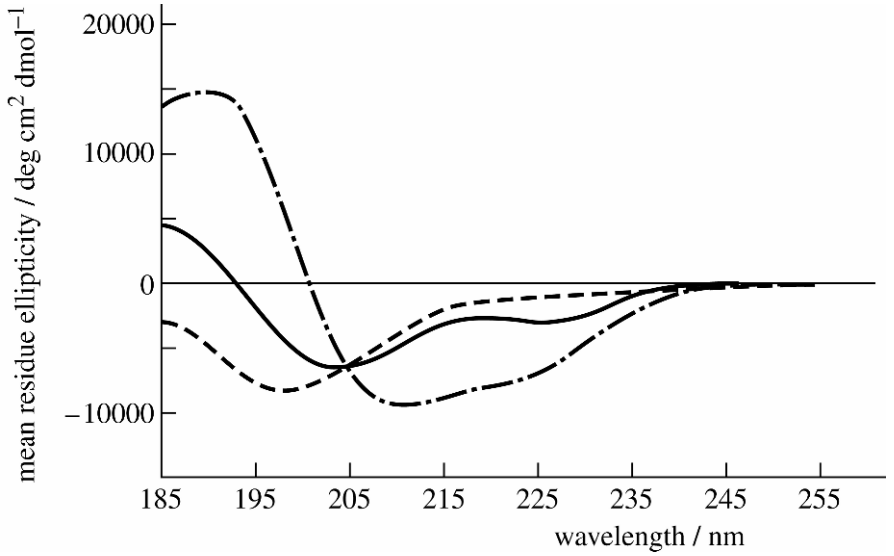

Fig. 10. Circular dichroism spectra of α-chymotrypsin in solution (——), adsorbed on Teflon (—·—·—), and on silica (— — —). Conditions as in Fig. 9. (Redrawn from Zoungrana and Norde 1997).

The addition of the $(EO)_8$-oligomers to the PS surface resulted in retention of some of the enzymatic activity of adsorbed α-chymotrypsin, whereas this activity was completely lost in the absence of the grafted oligomers. The short $(EO)_8$ chains trapped between the adsorbed protein molecules and the PS surface probably suppressed adsorption-induced structural perturbation and enzymatic inactivation. Because the surfaces in soil systems are, in most cases, hydrophilic rather than hydrophobic and as many of the surfaces may be covered with preadsorbed polymers or oligomers, it is expected that most proteins adsorbed at these surfaces retained, at least partly, their biological activity.

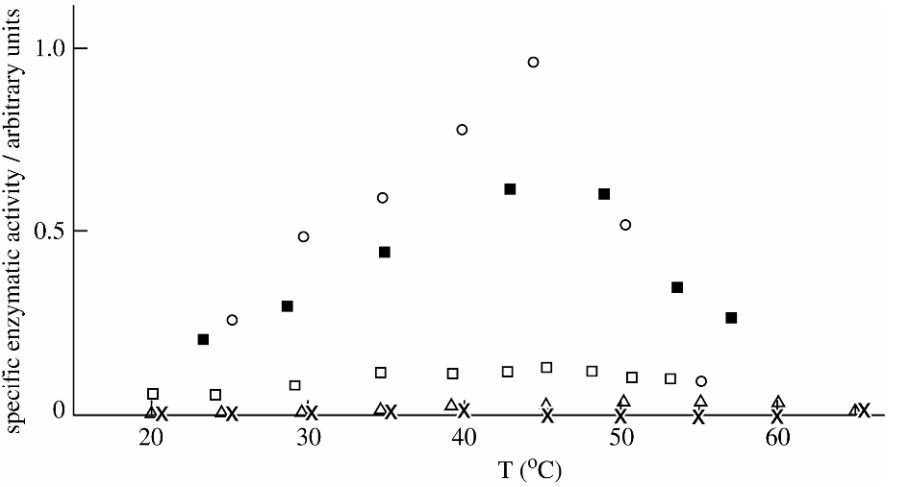

Fig. 11. Temperature dependency of the specific activity of α-chymotrypsin in solution (○), adsorbed on silica (■), Teflon (x), polystyrene (Δ) and polystyrene $-(EO)_8$ (□). Conditions as in Fig. 9. (Redrawn from Zoungrana and Norde 1997).

In this chapter the roles of various physico-chemical parameters in the interaction between globular proteins, e.g. enzymes, and soil minerals have been discussed semi-quantitatively. Knowledge of the mechanism of that interaction provides a basis to manipulate biological activity in soils.

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5 Relationship of Polarity and Structures of Organic Matter with Sorption Capacity for Hydrophobic Organic Compounds

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1 Introduction

Soil and sediment organic matter (SOM) comprises the sum total of organic materials in soil, including litter, microbial biomass, water-soluble organics, humic substances, and plant residues in varying stages of decomposition. Although the SOM content of mineral soils averages only 1–5%, SOM has a substantial impact on soil conditions (Stevenson 1994). For example, SOM is involved in the formation and stabilization of soil aggregates, promoting soil aeration and moisture retention. SOM is also the principal sorbent of hydrophobic organic compounds (HOCs), affecting their transport and bioavailability in soils and sediments (Xing and Pignatello 1998).

In 1968, researchers discovered that a moderate amount of SOM had a pronounced influence on the sorption of organic compounds, unless SOM content is very low (Lambert 1968). Due to the high affinity, the interaction of SOM with HOCs may be described by the following equation:

$$\log K_{oc} = a + b \log K_{ow}, \quad (1)$$

where K_{oc} is the organic carbon-normalized sorption coefficient (i.e., K_d/f_{oc}, where f_{oc} is the fraction of organic carbon in soil), K_{ow} is the octanol-water partition coefficient, and a and b are empirical constants. This equation has been widely used in predictive models for the movement and risk assessment of HOCs in soils and sediments, on the assumption that SOM behaves as a homogeneous partition phase, sorption occurs through partitioning, and octanol is an appropriate surrogate for SOM.

In reality, SOM is very heterogeneous in composition and structure. During sedimentation and diagenesis, biopolymers are degraded and cross-linked, forming humic substances (e.g., humic acids and humin) that may be further transformed into kerogen, coal, and graphite under metamorphic conditions. Within a single soil profile, the percentage of aromatic constituents in humic acids (HAs) increases as humification progresses with depth (Table 1). Furthermore, the chemical composition of HAs sequentially extracted from a single soil is quite variable (Kang et al. 2003).

The compositional and structural diversity of SOM leads to different sorptive properties for HOCs (Grathwohl 1990; Weber et al. 1992). For example, the sorption capacity of organic matter in unweathered shale and high-grade coals is more than an order of magnitude higher than that of organic matter derived from recent deposited soils, geologically immature material. Similar inferences can be drawn while comparing with the shale fraction of soils (Garbarini and Lion 1985; Grathwohl 1990). Furthermore, the measured K_{oc} values of PCBs and fluoranthene were quite different from the K_{oc} values calculated from equation (1) (Brannon et al. 1995). A number of investigators have reported that K_{oc} of HOCs is predominantly influenced by the chemical characteristics of SOM (Gunasekara and Xing 2003; Khalaf et al. 2003; Kulikova and Perminova 2002; Salloum et al. 2002; Xing 1997).

Nevertheless, a conclusive, distinct relationship between sorption of HOCs and the SOM characteristics has not been established. Here we summarize the literature data and our own findings on the correlation between HOC sorption and SOM characteristics with particular reference to the polarity of SOM.

2 Aromaticity of SOM and Sorption of HOCs

Solid state ^{13}C NMR spectroscopy has emerged as a very useful tool for characterizing SOM (Kinchesh et al. 1995; Preston 1996). Several workers have reported that K_{oc} of HOCs was linearly or exponentially related to the aromatic carbon contents of HAs or whole soils as determined by ^{13}C NMR analysis (Ahmad et al. 2001; Chen et al. 1996; Chin et al. 1997; Gauthier et al. 1987; Perminova et al. 1999). The K_{oc} value of humic materials can vary by as much as an order of magnitude, depending upon their origins (Fig. 1).

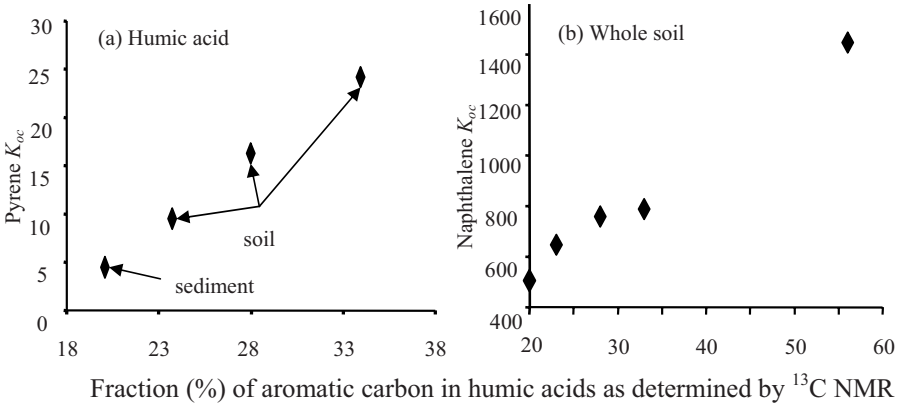

Fig. 1. Relationship between the K_{oc} of HOCs and the fraction of aromatic carbon in the sorbents as determined by ^{13}C NMR; (**a**) K_{oc} of pyrene for humic acids from three soils and a sediment as sorbents (Gauthier et al. 1987), (**b**) K_{oc} of naphthalene for five different whole soils as sorbents (Xing 1997).

Murphy and Zachara (1995) suggested that humic substances have heterogeneous sorption sites with those consisting of hydrophobic domains being strong and energetic. These domains may either be aromatic or aliphatic. Chen et al. (1996) reported strong sorption of α-naphthol by highly aromatic HAs, while Chin et al. (1997) found a linear relationship between the K_{oc} of HOCs and the aromaticity of HAs and non-extracted organic substances in whole soils and sediments. Similarly, Xing (2001) noted that the K_{oc} values for the sorption of phenanthrene by six HAs extracted from different depths within a single soil profile increased with the aromaticity of the sorbents (Fig. 2). Furthermore, an old and aromatic-rich organic matter, in coherence to the organic matter extracted from shale

or coal, yielded higher K_{oc} values than a young organic matter in surface soils (Fig. 1b). These observations indicate that the aromatic moieties in SOM, regardless of source, are the main sites for HOC sorption in soils and sediments.

Table 1. Structural carbon distribution (%) of the humic acids extracted from soil horizons, adopted from Xing (2001). The distribution was calculated from solid state ^{13}C Cross-Polarization Magic-Angle-Spinning (CP/MAS) NMR spectra. Chemical shift assignment for carbon functional groups: alkyl 0–50 ppm; O-alkyl 50–117 ppm; aromatic 107–165 ppm.

Horizon	Alkyl-C (%)	O-alkyl-C (%)	Aromatic-C (%)	Aromaticity (%)	Aliphaticity (%)
O1	24	29	28	35	30
O2	26	29	24	30	33
O3	25	30	25	31	31
A1	22	22	33	43	29
A2	13	16	47	62	17
A3	10	14	51	68	13

O, organic horizons; A, surface mineral horizons; 1–3, subhorizons.
Aromaticity = Aromatic C (107–165 ppm)/Sum of aliphatic C and aromatic C (0–165 ppm).
Aliphaticity = Aliphatic C (0–50 ppm)/Sum of aliphatic C and aromatic C (0–165 ppm).

The mechanism underlying the interaction of aromatic moieties in SOM with HOCs is yet to be clarified. One reason for this preferential interaction is the increased polarizability of the substrate in aromatic-rich humic substances (Chin et al. 1997; Gauthier et al. 1987). An increase in the polarizability of the humic materials may result in an increase in van der Waals interactions between the solute and substrate. In an aromatic-rich SOM, PCBs would be particularly susceptible to these interactions for congeners that possess weak dipole moments. For instance, Gauthier et al. (1987) ascribed the sorption enhancing effect of aromatic structures in SOM to a high polarizability and a favorable van der Waals interaction with polycyclic aromatic hydrocarbons (PAHs). The formation of charge-transfer complexes, where PAHs act as electron donors, was also discussed, while the aromatic moieties in SOM act as electron acceptors (Sander and Pignatello 2005; Zhu and Pignatello 2005). Chiou et al. (1998) attributed the enhanced partition of PAHs, as compared with other nonpolar solutes, to a better compatibility between the cohesive energy densities of PAHs and the

aromatic components in SOM. More recent reports suggest that the condensed aromatic structures as found in black carbon or soot carbon may also govern the sorption and distribution of HOCs in sediments (Accardi-Dey and Gschwend 2002; Bucheli and Gustafsson 2000). We will now discuss the importance of the aliphatic components of SOM to the sorption of HOCs.

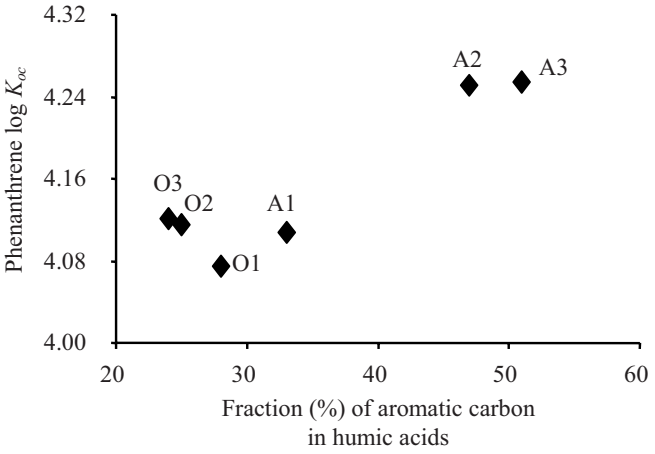

Fig. 2. Relationship between log K_{oc} of phenanthrene and the fraction of aromatic carbon in the humic acids extracted from soil horizons as determined by ^{13}C NMR. O, organic horizons; A, surface mineral horizons; 1–3, subhorizons. Modified from Xing (2001).

3 Aliphaticity of SOM and Sorption of HOCs

The aliphatic components of SOM, derived from various sources, tend to persist in soil (Almendros et al. 1998; Lichtfouse et al. 1998a; Lichtfouse et al. 1998b; Mosle et al. 1999; Poirier et al. 2000). The principal source of aliphatic materials in soil is plant cuticular materials, especially cutin, an insoluble polyester of cross-linked hydroxy-fatty acids and hydroxyepoxy-fatty acids (Kolattukudy 2001). Some plant cuticles also contain an acid and base hydrolysis-resistant biopolymer, comprised of aliphatic chains attached to aromatic cores known as cutan (Tegelaar et al. 1989; McKinney et al. 1996; Chefetz 2003; Sachleben et al. 2004).

Both cutin and cutan are difficult to degrade microbiologically, and could be selectively preserved in soils with little or no alteration (Almendros et al. 1996; Nierop 1998).

Hu et al. (2000) were able to detect semicrystalline poly(methylene) domains in natural organic matter (NOM) samples from various sources, resembling those in synthetic polyethylene. Relatively rigid crystalline layers of 3-nm thickness, with melting points around 75°C, were found adjacent to amorphous regions having rubber-like segmental mobility. Being resistant to microbial attack, the crystalline regions have long residence times, while the amorphous regions may play a role in the sorption of HOCs in soil.

Recent investigations have indicated that the aliphatic carbon fraction, rather than aromatic fraction, was strongly correlated to HOC sorption. For instance, the sorption of phenanthrene was related to nonpolar aliphatic carbon fraction, excluding poly(methylene), but was very strongly correlated with the content of the amorphous nonpolar aliphatic domains including amorphous poly(methylene) (Mao et al. 2002). In other words, the rubbery, relatively low-density, and amorphous nonpolar aliphatic carbon domains are excellent for phenanthrene partitioning (Figs. 3 and 4).

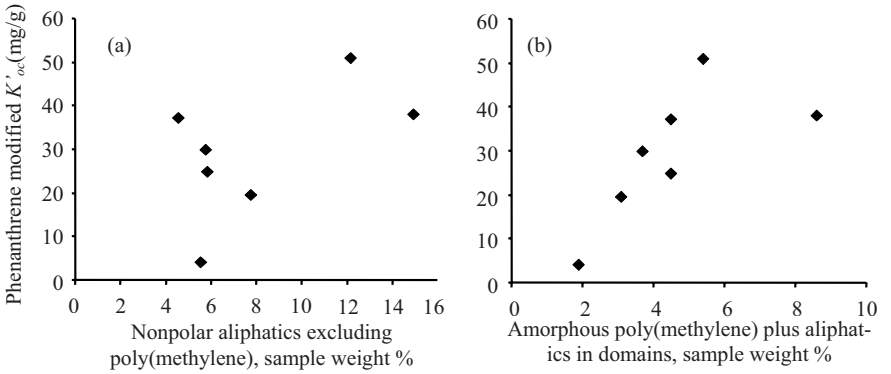

Fig. 3. Correlation of the modified Freundlich coefficient K'_{oc} with the weight fraction of (**a**) nonpolar aliphatic carbon, excluding poly(methylene) and (**b**) amorphous nonpolar aliphatic carbon in domains, including amorphous poly(methylene). Adapted from Mao et al. (2002).

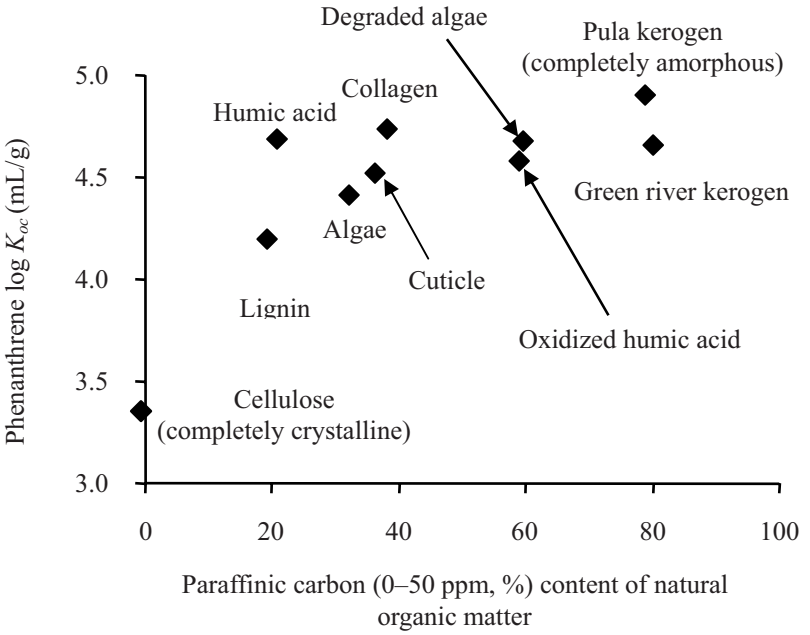

Fig. 4. Positive correlation between phenanthrene log K_{oc} values and paraffinic carbon content (0–50 ppm) of the natural organic materials calculated from CP/MAS ^{13}C NMR. Adopted from Salloum et al. (2002).

Chefetz et al. (2000) determined that cuticular plant material, composed mainly of aliphatic structures, sorbed more pyrene than the highly aromatic lignin and lignite. Also aliphatic corn leaf residues have high sorption affinity for HOCs (Boyd et al. 1990). Our study indicated that phenanthrene sorption was correlated with the aliphaticity of SOM (Table 2 and Fig. 5). Similarly, sorbents that contain a large amount of amorphous methylene carbon, such as the Pula kerogen sample (see Fig. 4), exhibited higher K_{oc} values for phenanthrene than the aromatic-rich samples (Table 3). On the other hand, the phenanthrene K_{oc} value for collagen was remarkably high, although this material does not contain polymethylenic carbon. This observation has been attributed to the ability of collagen to orient into a triple-helical structure with hydrophobic domains (Xing et al. 1994b).

Table 2. The aliphaticity and concentration-dependent organic carbon normalized sorption coefficient (log K_{oc}) for four humic acids (unpublished data by Kang and Xing, 2005)

Sample	Aliphaticity	(H+O)/C	Concentration-dependent log K_{oc}		
			C_e (μg mL^{-1}) = 0.005	C_e (μg mL^{-1}) = 0.05	C_e (μg mL^{-1}) = 0.5
F-1	0.63	0.607	4.17	4.05	4.01
F-4	0.69	0.570	4.57	4.41	4.25
F-7	0.70	0.559	4.61	4.42	4.34
F-9	0.72	0.486	4.72	4.52	4.37

F-1, F-4, F-7, and F-9 are the first, fourth, seventh, and nineth extracted humic acids , respectively.
Aliphaticity = Aliphatic C (0–108 ppm)/Sum of aliphatic C and aromatic C (0–162 ppm).
f_{oc} : Organic carbon content.
K_{oc} = (S/C_e)/f_{oc}; S is the solid-phase equilibrium concentration and C_e is the liquid-phase equilibrium concentration.

4 Polarity of SOM and Sorption of HOCs

During diagenesis, catagenesis, and coalification, NOM is subjected to chemical alteration, with the final product being possibly graphite - crystalline carbon (Allen-King et al. 2002). As a result of these processes, the hydrogen/carbon (H/C) atomic ratio, representing the aliphaticity of SOM, and the oxygen/carbon (O/C) atomic ratio, representing the polarity of SOM, generally decreased.

Grathwohl (1990) found a relationship between sorption capacity and the the atomic H/O ratio of NOM. Similarly, there is a good relationship between log K_{oc} and the polarity index (PI) of SOM, defined as the (O+N)/C ratio (DePaolis and Kukkonen 1997; Rutherford et al. 1992; Xing 1997; Xing et al. 1994a). The effect of SOM polarity on sorption of organic compounds is consistent with the well-known theory of solvent polarity on solute solubility. In studying the influence of SOM composition

on the partition of benzene and carbon tetrachloride, Rutherford et al. (1992) also demonstrated that K_{oc} values for both chemicals increased with decreasing polar content of organic sorbents. Recently, Wang and Xing (2005) demonstrated that adsorbed HAs onto montmorillonite and kaolinite were more hydrophobic and less polar, which favored phenanthrene sorption, resulting in a higher sorption capacity than the source HA. In our study (Fig. 6), the humin fractions with relatively low polarity as well as the later extracted HAs showed higher sorption capacity than the early extracted HAs with higher polarity, which reflects a negative relationship between polarity and log K_{oc}. Chen et al. (1996) reported that the K_{oc} of α-Naphthol is negatively related to the polarity of organic substances from soils and sediments (Fig. 7). Thus, with the previous results and our present data, we conclude that the polarity is one of the most important compositional parameters of SOM governing HOC sorption.

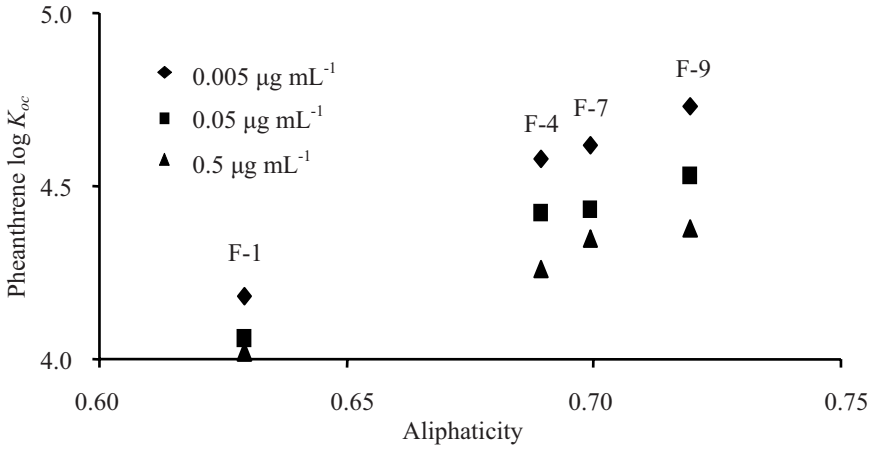

Fig. 5. Relationship between the log K_{oc} for phenanthrene sorption and the aliphaticity of humic acids, sequentially extracted from a soil. F-1, F-4, F-7, and F-9 are the first, fourth, seventh, and nineth extracted humic acids, respectively. 0.005, 0.05, and 0.5 μg mL^{-1} are selected liquid-phase equilbrium concentrations of phenanthrene (Kang and Xing 2005).

The use of solid state ^{13}C NMR spectroscopy along with elemental analysis provides useful information on the chemical composition of SOM samples. In assessing the influence of SOM composition on the partition of

benzene and carbon tetrachloride, Rutherford et al. (1992) founded that the K_{oc} values for both chemicals increased as the polarity of the organic sorbents decreased. The average K_{oc} calculated for carbon tetrachloride and 1,2-dichlorobenzene for sediments was twice as high as that for soils even though the samples did not differ appreciably in their aromatic carbon content as determined by solid state ^{13}C NMR (Kile et al. 1999). This finding indicated that the polar carbon content (alcoholic carbon plus carboxyl and carbonyl carbon) negatively affects sorption of the HOCs to organic matter.

Chiou et al. (1998) attributed the enhanced partitioning of PAHs with respect to other HOCs to relatively high compatibility between the cohesive energy densities of PAHs and the aromatic components in SOM. However, the difference in K_{oc} values between soils and sediments is related to the difference in polar group, rather than aromatic carbon, contents (Kile et al. 1999). The authors concluded that the content of polar groups (*O*-aryl and carboxyl C) has a large negative influence on K_{oc} values, and hence on HOC sorption in soil and sediment.

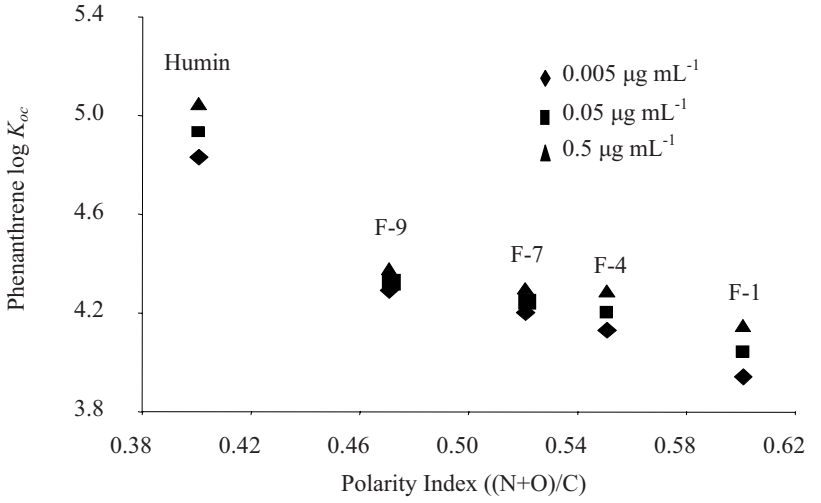

Fig. 6. Relationship between the log K_{oc} for phenanthrene sorption and the polarity index of humic acids and humin, sequentially extracted from a soil. F-1, F-4, F-7, and F-9 are the first, fourth, seventh, and nineth extracted humic acids, respectively. 0.005, 0.05, and 0.5 μg mL^{-1} are selected liquid-phase equilbrium concentration of phenathrene (Kang and Xing 2005).

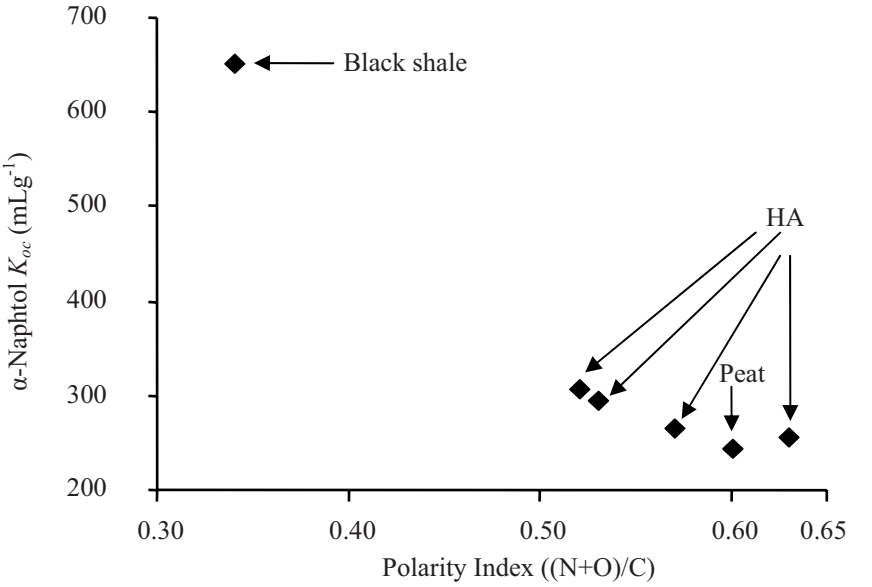

Fig. 7. Relationship between polarity index, (N+O)/C, and the K_{oc} of α-Naphthol sorption to peat, black shale, and humic acids (HA) from different horizons of a soil. Modified from Chen et al. (1996).

Table 3. Soil properties and K_{oc} of naphthalene (Xing 1997)

Adsorbent	Organic carbon content, %	Polarity Index, (N+O)/C	Aromatic carbon, %	K_{oc} of naphthalene (mL g^{-1})
Soil 1	53.4	0.51	56	1450
Soil 2	32.2	0.63	20	508
Soil 3	7.38	0.61	28	756
Soil 4	4.53	0.57	33	788
Soil 5	2.55	0.62	23	648

Aromatic carbon content for the soil materials was calculated from the CP/MAS ^{13}C NMR spectra using the ratio of peak area of 106–165 ppm to the total area of 0–230 ppm.

Although aromaticity, aliphaticity, and polarity have a significant influence on HOC sorption, it is difficult to determine which of these characteristics is predominant. Xing et al. (1994b) found K_{oc} to be highly

influenced by both polarity and aromaticity. For a number of soils, the polarity and proportion of aromatic carbon were strongly correlated, according to

$$PI = 0.702\ (\pm 0.0166) - 0.00353\ (\pm 0.00045)\ AR\ (r^2 = 0.969;\ p < 0.015862)$$

where AR = percent of aromatic carbon present as measured by CP/MAS ^{13}C NMR. Despite the small sample size, Fig. 8 summarizes the data on the relationship between the PI and percentage of aromatic carbon for several organic sorbents. The negative correlation suggests that polarity and aromaticity are interactive parameters regulating the sorption of HOCs. It appears that SOM with a high K_{oc} and aromaticity also have low polarity.

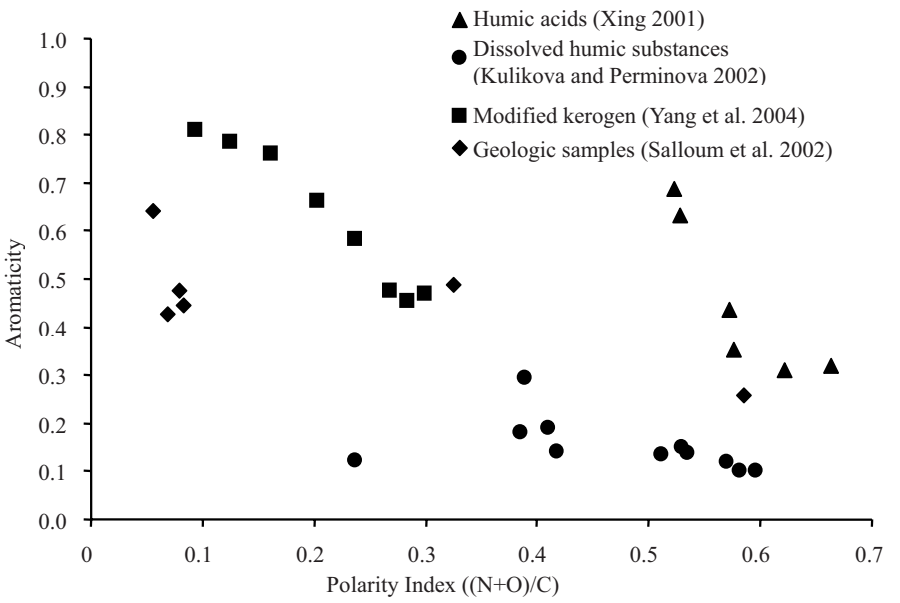

Fig. 8. Relationship between polarity index ((O + N)/C) and aromaticity of organic sorbents from the literature. The aromatic carbon was calculated as the product of aromatic carbon content (108–165 ppm) from NMR distribution and percentage of carbon contents from elemental analysis.

Whether aromatic or aliphatic groups are dominantly responsible for sorption of HOCs is still an open question (Kang and Xing 2005). High sorption of HOCs by both aliphatic-rich (Chefetz et al. 2000) and aromatic-rich SOM (Gauthier et al. 1987) has been reported. Kang and Xing (2005) observed that aliphatic-rich and aromatic-rich SOM with a relatively high K_{oc}, often has a low polarity. This type of correlation might be due to the dominant role of polar moieties in the samples for HOC sorption.

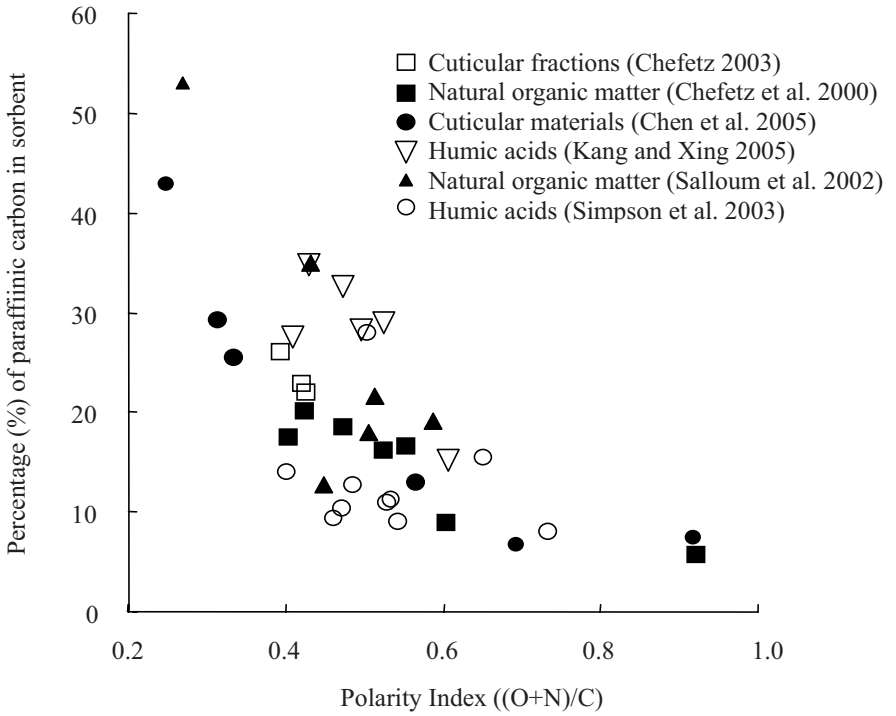

Fig. 9. Relationship between polarity index ((O + N)/C) and percentage of paraffinic carbon in organic sorbents from the literature. The percentage of paraffinic carbon was calculated as the product of paraffinic carbon content (0–50 ppm) from NMR distribution and percentage of carbon, nitrogen, and oxygen contents from elemental analysis.

A negative correlation was observed between PI and percentage of paraffinic carbon in organic sorbents (Fig. 9). This relationship is similar to the relationship between PI and aromaticity. Thus, polarity and paraffinic carbons, like polarity and aromaticity, could also be interactive

parameters regulating the sorption of organic pollutants. Therefore, sorbents with high K_{oc} (and aliphaticity) tend to have low polarity (Fig. 10). Thus, we believe that polarity of SOM appears to be the most dominant factor regulating the sorption of HOCs.

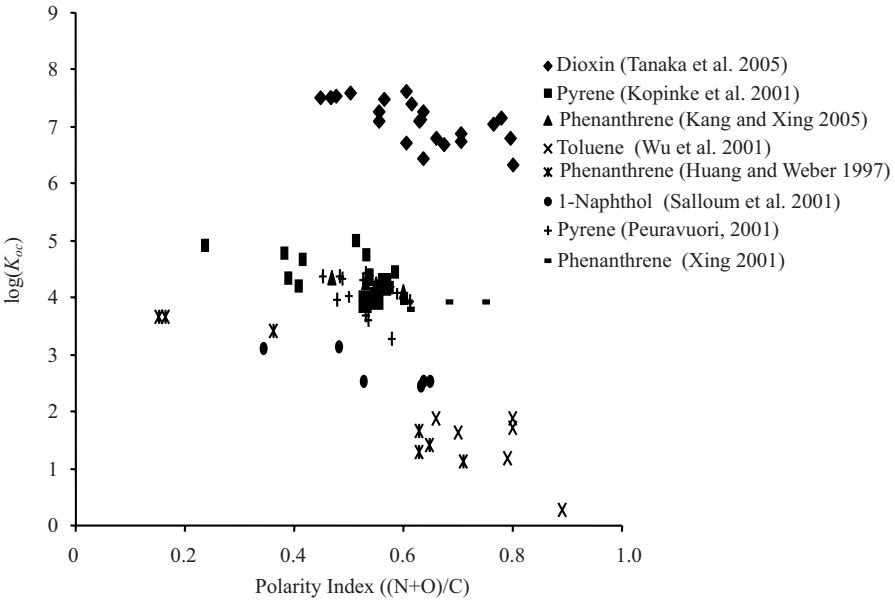

Fig. 10. Relationship between polarity index ((O + N)/C) and sorption capacity of HOCs from the literature.

5 Conclusions

The information addressed here emphasized that the HOC sorption capacity in context relates to the chemical structures of SOM. Aromatic structures of SOM were reported to be the domains primarily responsible for HOC sorption, supported by positive correlations between aromaticity and K_{oc}. Recently, aliphatic components, particularly paraffinic carbons, of SOM are reported to sorb significant amounts of HOCs, similarly supported by positive correlations between aliphaticity and K_{oc} values. From a series of sorption experiments and literature review, we concluded

that both aromatic and aliphatic components can be important indicators for the magnitude of K_{oc}. In addition, sorbents with high K_{oc} are often associated with low polarity whether their structures are highly aromatic or aliphatic.

Acknowledgments

We thank Saikat Ghosh and Elizabeth Johnson for their comments and suggestions. This work was in part supported by Research Grant No. IS-3385-03R from BARD, the United States – Israel Binational Agricultural Research and Development Fund, the CSREES, USDA National Research Initiative Competitive Grants Program (2005-35107-15278), and the Massachusetts Agricultural Experiment Station (MAS 90 and 8532).

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6 Organically Modified Clays for Pollutant Uptake and Environmental Protection

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1 Introduction

Because of their natural abundance and widespread occurrence, clays have been used 'in the service of man' (Konta 1995) since antiquity, notably as the raw materials in pottery and ceramics. Similarly, the use of fuller's earth (a calcium-rich montmorillonite) as a scouring and cleaning agent of raw wool dates back to before 2000 BC (Robertson 1986). Clays have also long served as medicinal and therapeutic agents, among which *Bolus Armenus* (a red clay from Cappadocia) and *terra sigillata* (a kaolinite-rich material from the island of Lemnos) are well known for their efficacy in curing festering wounds, skin afflictions, and snake bites (Robertson 1986,

Carretero et al. 2006, Droy-Lefaix and Tateo 2006). Likwise, Sudanese villagers along the Nile have traditionally used a local bentonite clay to rid river water of viruses and bacteria (Lund and Nissen 1986, Madsen and Schlundt 1989). For a description of the modern-day uses of clays and clay materials in various industries the reader is referred to the reviews by Murray (2003) and Harvey and Lagaly (2006).

Clay therapy and many practical applications of clays rely on the ability of these minerals to sorb and retain harmful and undesirable substances from their immediate environment. The sorptive capacity of clays is related to their small particle size, extensive surface area, layer structure, and charge characteristics. The vast literature on the interactions of clay minerals, especially smectites, with small and polymeric organic molecules has periodically been reviewed (Mortland 1970, Theng 1974, 1979, Huang and Schnitzer 1986, Yariv and Cross 2002, Lagaly et al. 2006). Although the reactivity of these minerals might be expected to extend to anthropogenic and industrial pollutants, the use of smectites for environmental protection is a relatively recent development. Its emergence is prompted by a growing awareness that industrial pollutants pose a threat to environmental and human health, and the need to find inexpensive and environmentally friendly materials for pollution control (Kowalska et al. 1994, Xu et al. 1997, Prost and Yaron 2001). Here we assess the literature that has accumulated over the past two decades on the use of smectites and their organically modified forms as sorbents of non-ionic organic compounds and pollutants. As far as is possible, we will refer to key papers and reviews, rather than cite individual authors.

2 Smectites and Modified Smectites

Smectites are 2:1 type phyllosilicates whose particles are made up of alumino-silicate layers stacked one on top of the other in a more or less regular array. Each layer consists of an alumina octahedral (O) sheet sandwiched between two opposing silica tetrahedral (T) sheets, forming a T-O-T layer structure. In such a structure there is scope for isomorphous substitution, i.e., the replacement of Al^{3+} in the octahedral sheet and/or Si^{4+} in the tetrahedral sheet by cations of similar size and coordination but of lower valency. As a result, the layers acquire a permanent negative charge which is balanced by exchangeable inorganic cations (e.g., Na^{+}, Ca^{2+}) occupying interlayer sites (Fig. 1, left). The magnitude of the layer charge (per formula unit) is reflected by the cation exchange capacity which typically ranges from 90 to 130 cmol/kg. Since the charge-balancing

cations or 'counterions' are normally hydrated, smectites are naturally hydrophilic. In the presence of water smectites are therefore not very reactive toward non-ionic organic compounds that are essentially hydrophobic, and do not compete well with water (Theng 1974).

We should mention, however that under certain conditions some smectites can sorb appreciable amounts of non-ionic organic compounds from aqueous solutions. Laird et al. (1992), for example, observed that Ca^{2+}-smectites could take up atrazine from water under mildly acidic pH conditions. Since sorption affinity was inversely related to the layer charge density of the minerals, they suggested that the neutral form of atrazine was sorbed through hydrophobic interactions with "uncharged" regions on basal siloxane surfaces. Similarly, smectites exchanged with cations of low hydration energy (K^+, NH_4^+) could sorb a variety of nitroaromatic compounds from water through such mechanisms as electron donor-acceptor complex formation (Haderlein et al. 1996, Weissmahr et al. 1997) and cation-organic solute interactions (Boyd et al. 2001, Johnston et al. 2001).

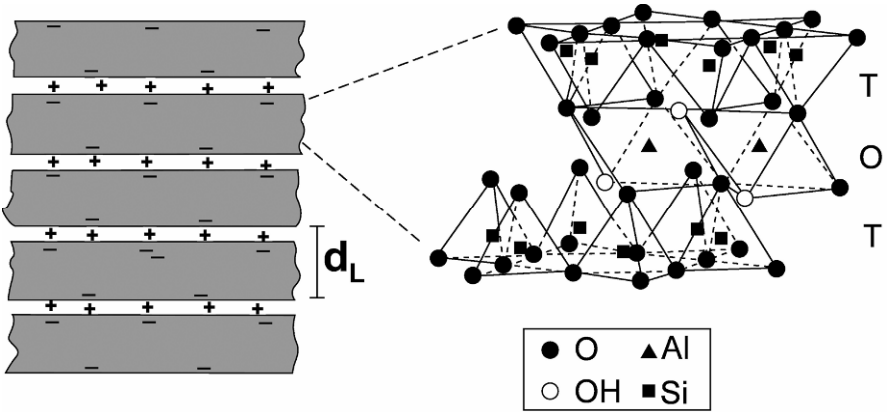

Fig. 1. Diagram showing the layer structure of 2:1 type clay minerals as in smectites. *Left*: a particle comprising five layers stacked one on top of the other; the negative layer charge (arising from isomorphous substitution) is balanced by inorganic cations occupying interlayer sites; d_L = basal spacing. *Right*: detail of the atomic arrangement in an individual T-O-T layer where T denotes a tetrahedral sheet, and O represents an octahedral sheet. After: Lagaly and Köster (1993).

The surface properties and reactivity of smectites may be modified by mechanical, physical, and chemical means. Grinding (manual and percussive) is a long-standing mechanical method of modification (Grim 1968, Čičel and Kranz 1981). Thermal treatment (heating) is an oft-used physical method of altering the surface area, porosity, swelling, and water dispersibility of smectites (Chorom and Rengasamy 1996, Bojemueller et al. 2001). At sufficiently high temperatures dehydroxylation occurs, causing changes in the structure of the octahedral sheet followed by the formation of new, high-temperature phases (Heller-Kallai 2006).

Acid activation and 'pillaring' are two common chemical methods for inducing surface modification. As the name suggests, 'acid activation' involves treating clays (usually calcium-rich bentonites) with a solution of HCl or H_2SO_4 for a specified period, yielding materials of high surface area, mesoporosity, and surface acidity (Christidis et al. 2003, Churchman and Volzone 2003, Komadel and Madejová 2006). Acid-activated clays and their natural counterparts ('bleaching earths') are widely used in decolourising edible oils and animal fats (Anderson and Williams 1962, Siddiqui 1968, German Society for Fat Science 2001, Christidis et al. 2003). These materials can also function as catalysts for organic conversions, and carrriers of fungicides and insecticides (Breen et al. 1997, Komadel and Madejová 2006). However, acid-activated clays have not widely featured as sorbents of non-polar organic compunds. 'Pillaring' involves the replacement of the inorganic counterions (Na^+, Ca^{2+}) with oligomeric (hydr)oxy metal cations, followed by heating ($\geq 300^oC$). This converts the oligomeric cations into the corresponding metal oxides acting as nano-size pillars in the interlayer space. Besides being efficient sorbents of organic compounds, micro- and meso-porous 'pillared interlayered clays' are useful as catalysts, molecular devices and sensors (Zielke et al. 1989, Mitchell 1990, Adams and McCabe 2006, Bergaya et al. 2006).

Perhaps the single, most common method of modifying the surface and sorptive properties of smectites is by intercalation of simple and polymeric organic cations through an ion-exchange process (Theng 1974, 1979). The formation of some polycation-exchanged smectites together with their relative efficiency in taking up non-ionic organic compounds from water have been summarised by Breen (1999) and Churchman and Volzone (2003), and will not be further discussed. Rather, the focus here is on smectites intercalated with simple organic cations of which two types may be distinguished. Type I are formed by replacing the inorganic counterions in smectites with short-chain, compact alkylammonium or quaternary ammonium ions, such as tetramethylammonium, tetraethylammonium, and trimethylphenylammonium. Organically modified smectites of

type II are obtained by intercalation of long-chain alkylammonium ($[H_3N]R^+$) or quaternary ammonium ($[CH_3]_3NR^+$) ions where R represents an alkyl chain. The demarcation between short- and long-chain quaternary ammonium ions will be described later. In the literature the first type of organically modified smectites are often referred to as 'adsorptive clays' (e.g., Brixie and Boyd 1994), while the term 'organoclays' is used to denote the second type. Here we use 'organoclays' as a general term for smectites that have been exchanged or intercalated with simple, non-polymeric organic cations.

3 Organoclays

3.1 General

The ability of smectites, especially montmorillonites, to take up positively charged organic compounds has been known for nearly a century (Theng 1974). It was not until the 1950s, however, that the formation, properties, and practical applications of organically modified smectites began to be systematically investigated (Weiss 1963, Theng 1972, Barrer 1978, Lagaly et al. 2006). Although cation exchange is the principal mechanism underlying the adsorption of alkylammonium ions by montmorillonite, van der Waals interactions between the alkyl chain and the silicate surface can contribute appreciably to the overall adsorption energy (Theng et al. 1967, Vansant and Uytterhoeven 1972). The extent of this contribution increases with the molecular weight (size) of the organic cations.

Intercalation of short-chain, compact alkylammonium ions gives rise to type I complexes with a basal spacing of about 1.5 nm, corresponding to an interlayer distance of ~0.55 nm. As a result, a permanent interlayer free space (between the organic cations) is created into which various non-ionic organic compounds can be accommodated. The volume of this space is controlled by the interlayer distance and (negative) layer charge separation of the smectite (Fig. 2). The sorption capacity of type I organoclays is also dependent on the size of the intercalated alkylammonium ion since the bulkier the organic cation the larger the fraction of interlayer space it occupies.

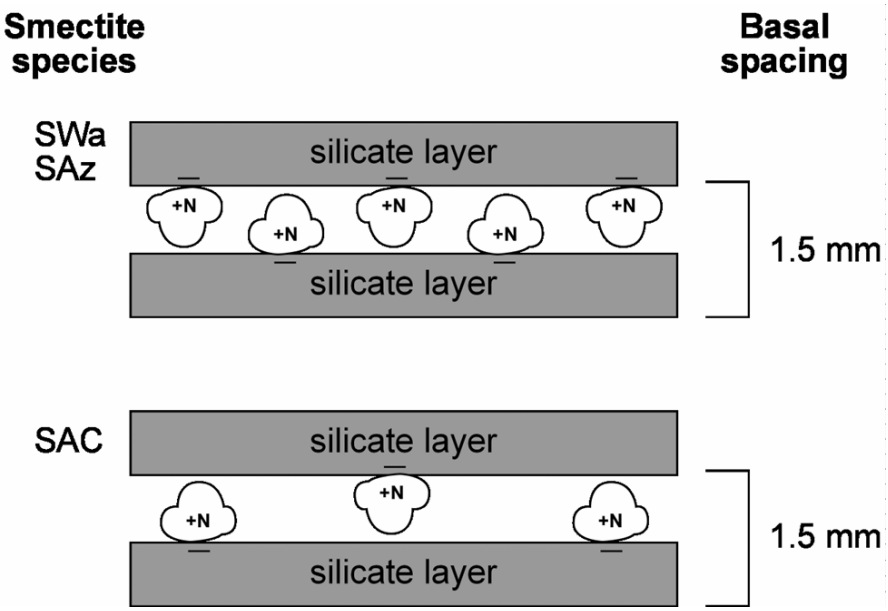

Fig. 2. Diagram showing the intercalation of compact quaternary ammonium cations, such as trimethylphenylammonium (TMPA) into different smectites, giving rise to type I organoclays with a basal spacing of about 1.5 nm. SWa is a high-charge nontronite (iron-rich smectite) and SAz is a high-charge montmorillonite, while SAC is a low-charge montmorillonite. After: Jaynes and Boyd (1991b).

Type II organoclays, on the other hand, have a very limited interlayer free volume since much of the interlayer space is occupied by the more or less flat-lying long-chain alkylammonium or quaternary ammonium ions. Here the arrangement of the intercalated organic cations is strongly dependent on both the alkyl chain length (n_c) and the layer charge of the clay mineral. A stable monolayer arrangement (basal spacing = 1.37 nm) is obtained for n_c = 11–13 when the area of the alkylammonium cation is smaller than the area per exchange site. When area occupied by the organic cation exceeds the area per exchange site, a bilayer arrangement may be obtained, and the basal spacing increases to 1.77 nm. Thus, as the layer charge increases the transition from monolayer to bilayer occurs at a smaller n_c value (shorter chain length). Indeed, a pseudo-trilayer arrangement (basal spacing ~ 2.2 nm) obtains with high-charge smectites and/or large n_c values. With highly charged 2:1 layer silicates (e.g., vermiculites), the paraffin-type arrangement is preferred

giving rise to large basal spacings (> 2.2 nm) whose value increases regularly with n_c (Lagaly 1982, Jaynes and Boyd 1991a, Slade and Gates 2004b, Lagaly et al. 2006). The different interlayer arrangements of long-chain alkylammonium cations in smectites are illustrated in Fig. 3.

Because of their thixotropic gelling properties type II organoclays have long been applied as thickeners in lubricants, ointments, and paints. More recently, these materials have attracted great attention and interest for the synthesis of organoclay-polymer nanocomposites, a novel class of inorganic-organic hybrid materials with superior mechanical, thermal, and gas-barrier properties (LeBaron et al. 1999, Ahmadi et al. 2004, Ruiz-Hitzky and Van Meerbeek 2006). The present article, however, is concerned with using organoclays as sorbents of non-ionic organic compounds many of which are pollutants from agricultural and industrial sources.

3.2 Uptake of Non-Ionic Organic Compounds (NOCs)

Research into the formation, properties, and reactivity of type I organoclays was pioneered by Barrer and co-workers in the 1950s (see Barrer 1978). These materials can take up appreciable amounts of various aliphatic and aromatic hydrocarbons as well as small polar molecules (ammonia, methanol) from the gas phase. The guest molecules are accommodated within the interlayer free space between individual quaternary alkylammonium ions (cf. Fig. 2). Since tetramethylammonium (TMA)-montmorillonite has a larger interlayer porosity than the tetraethylammonium (TEA)-exchanged form, its capacity for taking up a given NOC is correspondingly greater. Likewise, less of the NOC is sorbed by a high-charge smectite, exchanged with TMA ions, than by the corresponding complex with a low-charge mineral (Lee et al. 1990). By contrast, sorption of gaseous NOCs by a type II organoclay, such as hexadecyltrimethylammonium (HDTMA)-smectite, tends to increase with the amount of HDTMA intercalated. Furthermore, the sorption isotherm tends to be non-linear at fractional 'loadings' by HDTMA (i.e., less than the CEC of the clay mineral) but becomes essentially linear at 100% exchange (Boyd et al. 1988a, Zhu and Su 2002). This would indicate that NOCs are taken up by both solute-surface interactions and solute partitioning into the interlayer HDTMA phase. The latter process predominates as the interlayer space fills up with HDTMA.

Churchman et al. (2006) have proposed that smectite complexes with quaternary ammonium ions (QACs) containing fewer than 9 carbon atoms dominantly take up NOCs by (chemical) interaction with the interlayer surface. This process is characterised by non-linear isotherms and competitive uptake when more than one solute is present. On the other hand, uptake of NOCs by complexes with QACs having more than 14 carbon atoms dominantly occurs by partitioning (Chiou 1989). Here the interlayer QACs (cf. Fig. 3) essentially act as a solvent for the NOCs, giving rise to linear isotherms and non-competitive solute uptake. Although this 'working hypothesis' is supported by experiment (e.g., Smith et al. 1990, Jaynes and Boyd 1991a), the reality is a little more complex than what is outlined above (Jaynes and Vance 1996, 1999, Sheng et al. 1996a, Singh et al. 2003).

Indeed, it is not uncommon to observe 'double-sigmoid' isotherms for the sorption of NOCs by type II organoclays, especially in the case of low-charge smectites and at low solute concentrations (Sheng et al. 1996b). Combining a sigmoid and a type III isotherm, the double-sigmoid isotherm characterises the sorption of a number of aromatic and substituted aromatic compounds (e.g., benzene, nitrobenzenes, and chlorobenzenes) by HDTMA smectites. In explanation, Sheng et al. (1996b) propose that such molecules interact strongly with HDTMA through a variety of mechanisms, including solvation of the cationic ammonium centres and the alkyl chains of HDTMA, and solute partitioning. As a result, the HDTMA chains re-orient from a parallel to a more vertical position with respect to the silicate surface, and interlayer swelling occurs. The behaviour of aromatic contaminants at organoclay surfaces will be further discussed later.

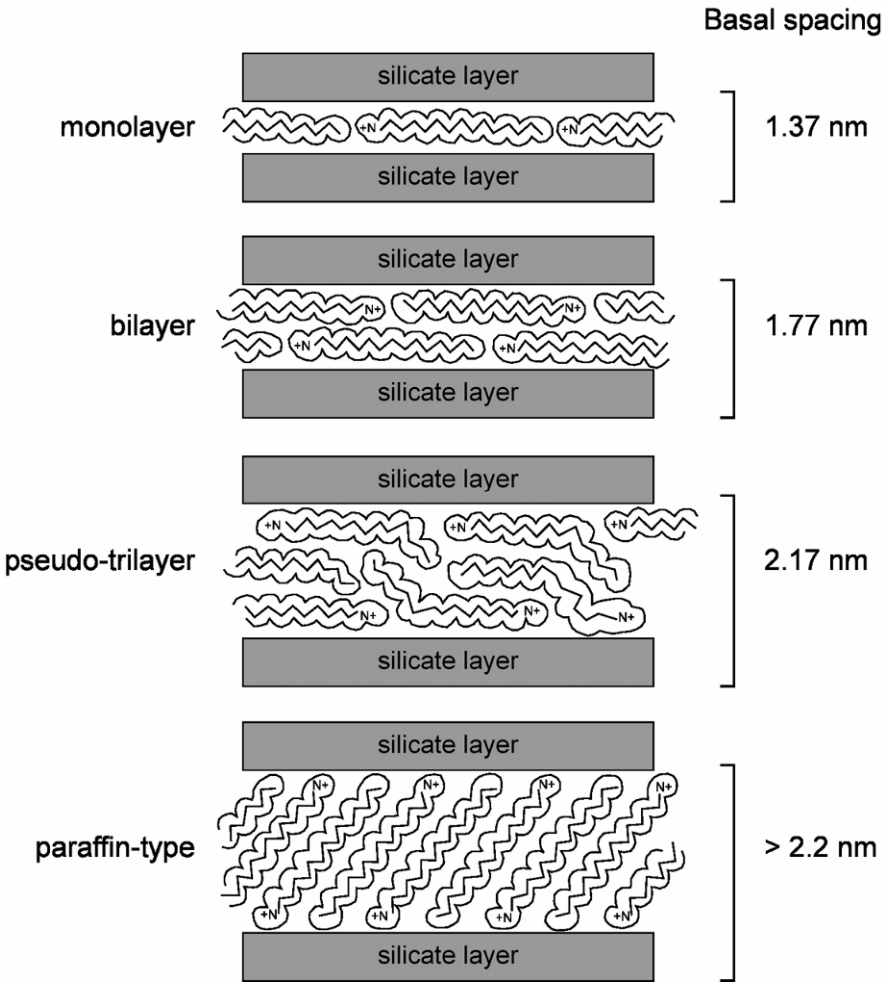

Fig. 3. Possible arrangements of long-chain quaternary ammonium cations (here tetradecyltrimethylammonium) in the interlayer space of expanding 2:1 layer silicates. After: Lagaly (1982) and Jaynes and Boyd (1991a).

In terms of pollution control, however, it is the capacity of organoclays to take up NOCs from aqueous solutions (rather than the gas phase) that has been the focus of attention since the early work by Cowan and White (1962) and Street and White (1963). In general, type II organoclays can sorb more NOCs from water than their type I counterparts (Mortland et al. 1986, Boyd et al. 1988b). On the other hand, type I organoclays can show a zeolite-like selectivity for sorption (Sharmasarkar et al. 2000, Shen 2002). Besides being dependent on the dimension of the intercalated QACs, selectivity is also influenced by the size and shape of the NOCs. For example, Lee et al. (1989a) found that the herbicide lindane (hexachlorocyclohexane, γ-isomer or γ-BHC) was effectively excluded from the interlayers of tetramethylammonium-smectite. Selectivity also depends on the extent of 'loading' by the QAC as well as the presence of water (Kukkadapu and Boyd 1995, Sheng and Boyd 1998). This is because the volume of the interlayer free space decreases as more and more exchange sites become occupied by the QAC, and extent of hydration of the organic cation increases.

For type II orgamoclays the uptake of NOCs generally increases as the organic carbon (QAC) content of the complexes rises (Hassett and Banwart 1989, Kowalska et al. 1994, Redding et al. 2002, Slade and Gates 2004b). This is because uptake essentially occurs by partitioning into the interlayer organic (QAC) phase. An earlier example is provided by Jaynes and Boyd (1991a) who measured the uptake from water of 8 aromatic NOCs by HDTMA complexes with 7 layer silicates, including some smectites. Figure 4 shows the uptake of ethylbenzene by 3 organically modified smectites with varied cation exchange capacity (CEC), and where all the exchange sites are occupied by HDTMA ions. Sorption increases with the CEC (i.e., HDTMA content) of the smectite, while the Mg^{2+}-exchanged form (without HDTMA) is inactive. The linear shape of the isotherms is strongly indicative of a partitioning process.

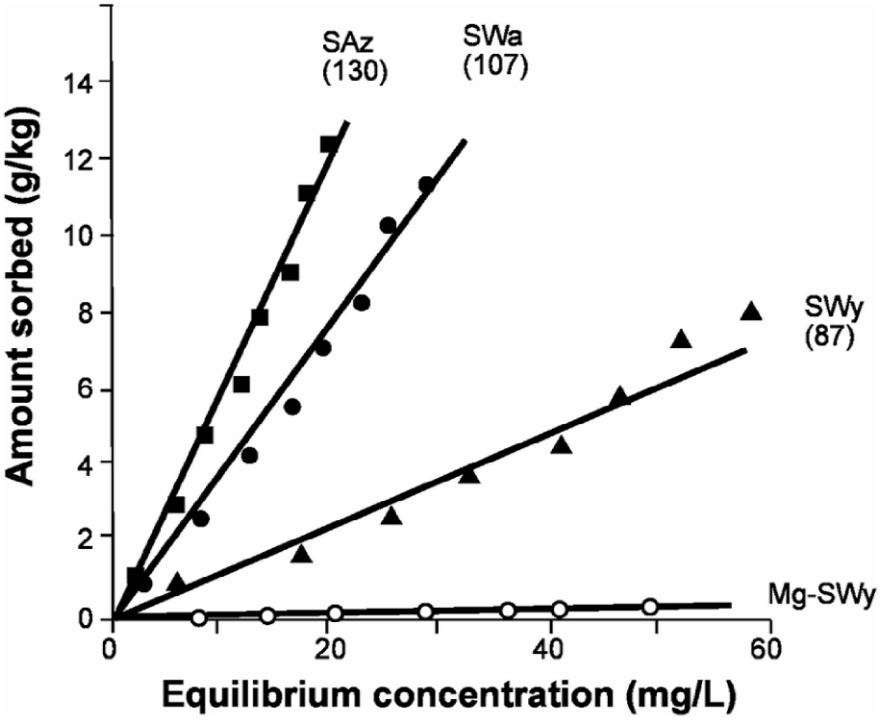

Fig. 4. Isotherms for the uptake of ethylbenzene from water by different smectites intercalated with hexadecyltrimethylammonium (HDTMA) ions. Numbers in brackets refer to the cation exchange capacities (CECs) of the smectites. Both SAz and SWa are high-charge smectites (cf. Fig. 2). SWy is a relatively low-charge montmorillonite, and Mg-SWy represents the corresponding Mg^{2+}-exchanged form. After: Jaynes and Boyd (1991a).

Another feature of the process is that the sorption capacity of type II organoclays is inversely related to the aqueous solubility of the NOCs (Chiou 1989). For example, the affinity of HDTMA-smectite for various phenols increases in the order: phenol < chlorophenol < dichlorophenol < trichlorophenol since phenol is the most water-soluble while trichlorophenol is the most hydrophobic (Mortland et al. 1986, Lo et al. 1998). The relationship between the distribution (partition) coefficient in a type II organoclay and water-solubility is illustrated in Fig. 5 for a range of nonionic organic pollutants.

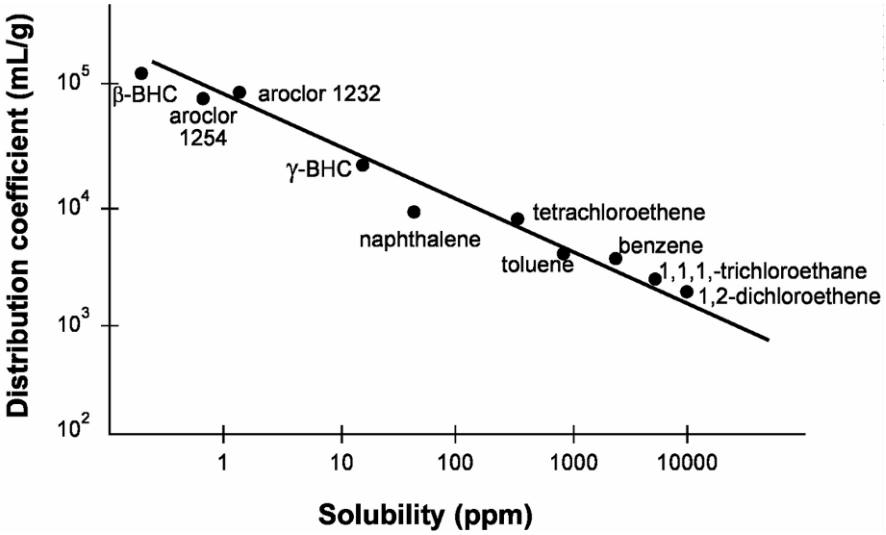

Fig. 5. Relationship between the distribution (partition) coefficient on dimethyl dihydrogenated tallow montmorillonite for a range of non-ionic organic pollutants and their corresponding solubility in water. BHC is benzene hexachloride, the γ-isomer of which is known as 'lindane'; aroclor 1232 and aroclor 1252 denote mixtures of polychlorinated biphenyls containing about 32 and 52% chlorine, respectively. After: Beall (2003).

3.3 Uptake of Organic Pollutants

The uptake of non-ionic organic pollutants by type II organoclays may be enhanced by increasing: (i) the amount of intercalated quaternary ammonium cations (QACs) and ion-pairs (the QAC cation plus its inorganic anion); (ii) layer charge density of the smectite; and (iii) the length of the alkyl chain (n_c) of the QAC (Cowan and White 1962, Boyd et al. 1988b, Jaynes and Boyd 1991a, Lee et al. 2004, Slade and Gates 2004b). The structure of the QAC can also affect sorption. For example, in examining the uptake of 'BTEX' (benzene, toluene, ethylbenzene, xylene) pollutants from water by different organoclays, Jaynes and Vance (1996) observed that uptake was generally proportional to the carbon content of the organoclay. However, the smectite intercalated with dodecyltrimethylammonium (n_c = 15; molecular weight = 228) was much more effective in taking up BTEX than the complex with cyclododecyltrimethylammonium (n_c = 15; molecular weight = 226). They also found that the uptake of a given BTEX component from the mixed solution was enhanced relative to that of the corresponding 'pure' compound. This process of 'co-sorption' in which uptake of a particular pollutant is enhanced by another species often occurs

as a result of QAC solvation by one of the co-sorbates. Thus, the enhanced sorption by hexadecyltrimethylammonium (HDTMA)-smectite of trichloroethylene, in the presence of nitrobenzene or chlorobenzene, is apparently due to the solvation of interlayer HDTMA by the aromatic compounds. This brings about a re-alignment of HDTMA, creating more interlayer free space for sorption of trichloroethylene (Sheng et al. 1996a, b). Such a mechanism has also been shown to occur for the uptake of toluene by low-charge HDTMA-vermiculite intercalates (Slade and Gates 2004a).

As already remarked on, the interlayer (siloxane) surface of smectites are actively involved in the adsorption of alkylammonium ions (Theng et al. 1967). The work by Jaynes and Boyd (1991b) further indicates that this surface is essentially hydrophobic. Thus, when smectites with varied layer charge are exchanged with trimethylphenylammonium (TMPA) ions, the resultant organoclays (type I) developed an appreciable capacity for taking up hydrocarbons (benzene, alkylbenzenes, naphthalene) from water. Morevover, the amounts sorbed decrease as the cation exchange capacity (i.e., the TMPA content) of the smectites increases (Fig. 6). This observation and the curvilinear shape of the isotherms strongly suggest that the sorbed hydrocarbon molecules interact with the oxygens of the siloxane surface, exposed between individual TMPA ions in the interlayer space (cf. Fig. 2). It would therefore appear that the hydrophilicity of natural smectites is not an intrinsic surface property but, rather, is due to the presence of hydrated inorganic counterions.

In this context we might add that the hydration energy of the exchangeable cations is a key factor influencing the competition for surface sites between water and potentially sorbed non-ionic organic molecules. Exchangeable cations of high hydration energy would retain water preferentially, while cations of low hydration energies would prefer organic solutes to water. As already remarked on, K^+- and Cs^+-exchanged smectites (of low to moderate layer charge density) can have a high affinity for certain non-ionic organic molecules (e.g. nitroaromatics) in the presence of water. The current theory proposed (Jaynes and Boyd 1991b, Laird et al. 1992, Boyd and Jaynes 1994) to explain this behaviour is that cations of low hydration energy can effectively compensate for the negative layer charge of smectites. As a result, the interlayer siloxane surface between isomorphous substitution sites becomes less negatively charged and more hydrophobic than would otherwise be the case (Schoonheydt and Johnston 2006). Some interlayer water, however, is required to initiate crystalline swelling, enabling organic molecules to penetrate the interlayer space (e.g. Chappell et al. 2005).

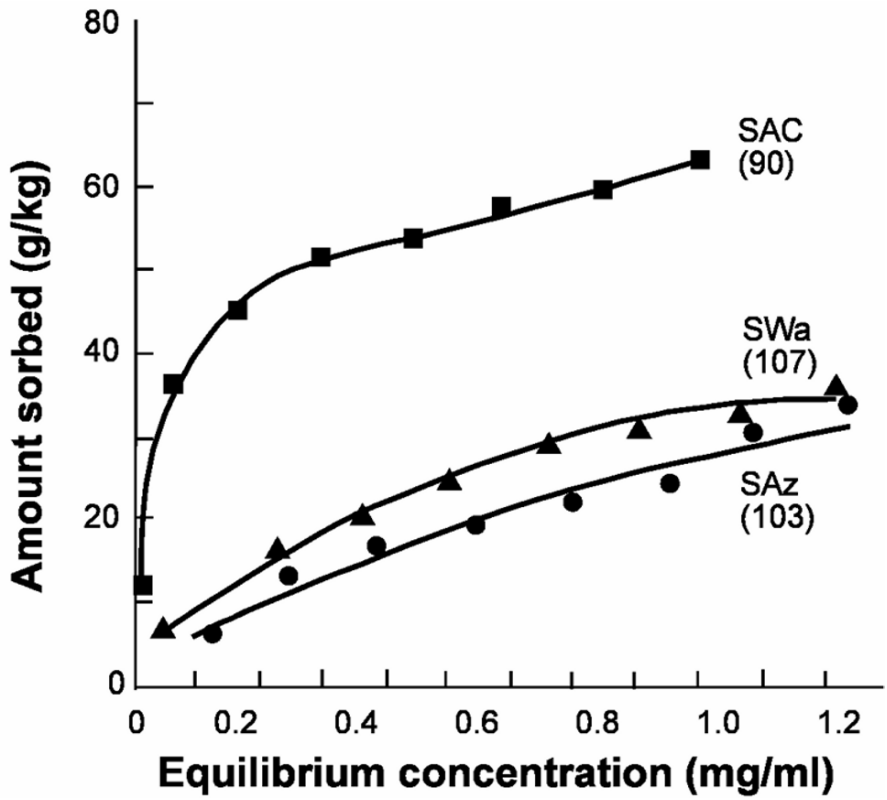

Fig. 6. Uptake of benzene from water by various smectites exchanged with trimethylphenylammonium (TMPA) ions (cf. Fig. 2). Numbers in brackets refer to the cation exchange capacities of the smectites. (cf. Fig. 4). After: Jaynes and Boyd (1991b).

In recognising that organoclay liners may usefully control wastes containing different proportions of organic solvents, Nzengung et al. (1996) have measured the uptake of two NOCs (naphthalene and diuron) from mixed methanol-water solutions by types I and II organoclays. The presence of methanol is conducive to uptake. This is because methanol can swell the organoclays, making their interlayers more organophilic and accessible to NOCs than otherwise would be the case (Moraru 2001). Being more aromatic than diuron, naphthalene is more strongly attracted to TMPA-montmorillonite than to the tetramethylammonium-exchanged form. This is because the aromatic ring of naphthalene and diuron can interact with that of TMPA through π-π bonding. In addition, steric effects come into play in that the relatively small naphthalene molecule can enter into the interlayer space of the organoclay whereas this is not possible

for the bulky diuron. Thus, although diuron has a benzene ring, its size and/or shape 'probably prevented the specific favorable interaction between its ring structure and a sorbent's benzene ring' (Nzengung et al. 1996). Similarly, interaction between the phenyl ring of some pesticides (e.g. acetochlor, alachlor, metolachlor, norflurazon) and TMPA has been proposed to account for the enhanced sorption of these compounds by TMPA-montmorillonite in comparison with a type II organoclay (Nir et al. 2006). The use of organoclays in pesticide formulations will be described later.

The efficiency of type II organoclays in taking up organic pollutants from water is also apparent from the data in Figs. 7 and 8 showing the removal of naphthalene and 17β-estradiol (an endocrine-disrupting compound) by octadecyltrimethylammonium-montmorillonite (Yuan 2004).

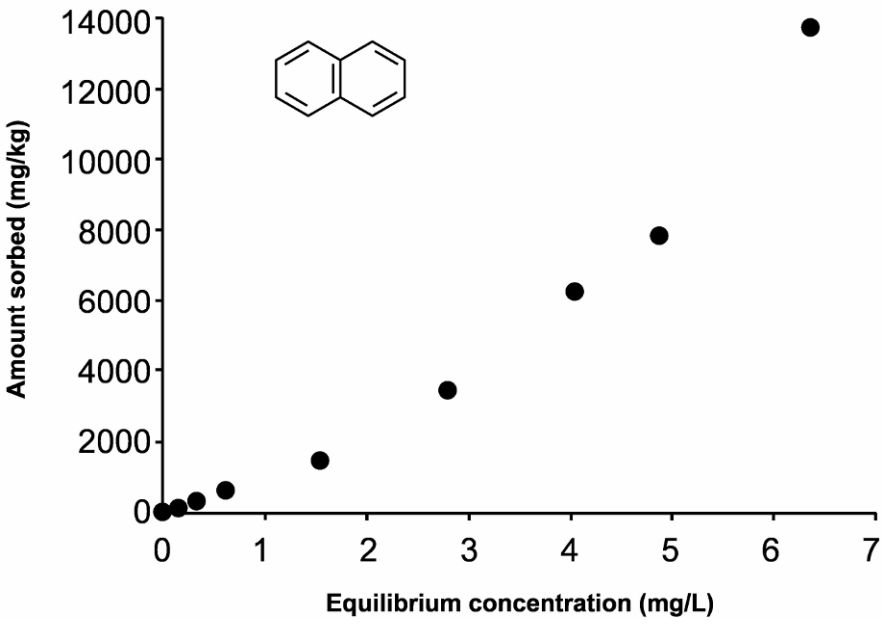

Fig. 7. Isotherm for the uptake of naphthalene (from water) by octadecyltrimethylammonium (ODTMA)-montmorillonite. After: Yuan (2004).

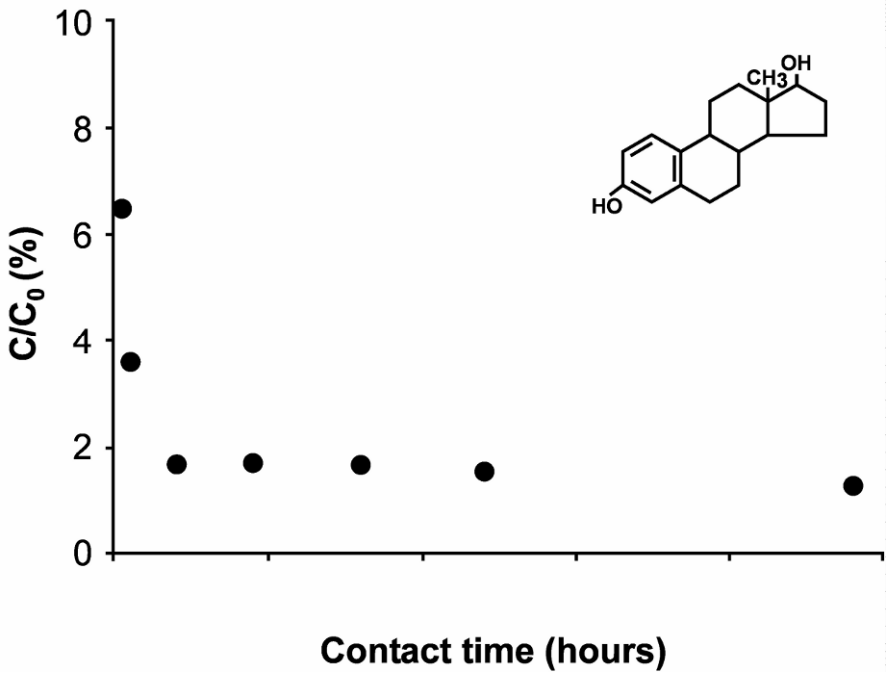

Fig. 8. Depletion of 17β-estradiol from aqueous solution after contact with octadecyltrimethylammonium (ODTMA)-montmorillonite. C denotes the concentration of the estrogen after a given contact time, while C_0 is the initial concentration. After: Yuan (2004).

We should also mention that type II organoclays can incorporate large amounts of polycyclic aromatic hydrocarbons (PAHs) from the solid phase (Ogawa et al. 1992). For example, Theng et al. (1998) were able to intercalate up to ⅓ by weight of solid phenanthrene (Ph) into a dry bilayer complex of tetradecyltrimethylammonium (TDTMA)-montmorillonite (cf. Fig. 3) by grinding the two solid components together. As a result, the basal spacing nearly doubles from about 1.8 nm (for the initial TDTMA complex) to near 3.4 nm. This would indicate a conformational change of TDTMA ions from flat-lying to one in which the alkyl chains extend away at a high angle from the interlayer surface. Solid-state ^{13}C-nuclear magnetic resonance spectroscopy shows that intercalation of Ph into TDTMA-montmorillonite causes a displacement by nearly −3 ppm of the $-(CH_2)_n-$ signal for TDTMA. This signal and that for intercalated Ph are also broadened relative to the corresponding pure compounds. Furthermore, the proton spin relaxation time constant,

$T_1(H)$, for intercalated Ph is more than four orders of magnitude shorter than that for the pure compound. Equally striking is the close similarity of the $T_1(H)$ value between TDTMA and Ph in the TDTMA-montmorillonite-Ph interlayer complex. All these observations strongly indicate that the TDTMA chains become relatively disordered and closely mixed with Ph in the interlayer space of montmorillonite.

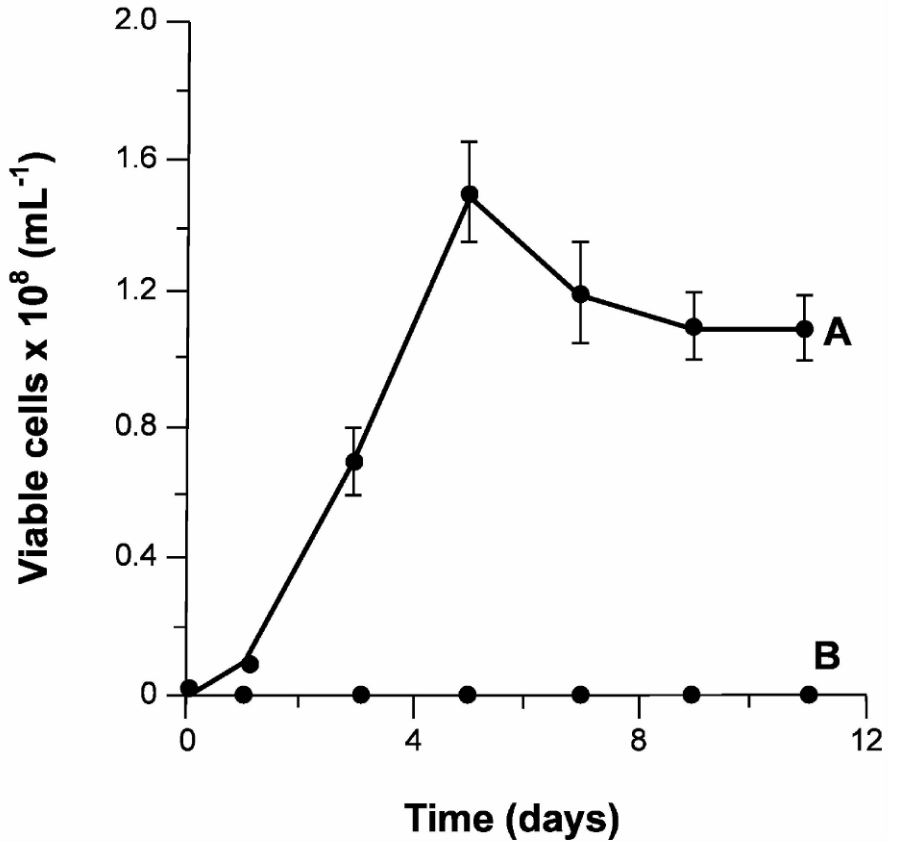

Fig. 9. Growth of phenanthrene-degrading bacteria (*Burkholderia* sp. RP007) in the presence of either phenanthrene in the free form (**A**), or phenanthrene intercalated into tetradecyltrimethylammonium (TDTMA)-montmorillonite (**B**). After: Theng et al. (2001).

The intercalated phenanthrene is not bioavailable, at least over the 11-day period of incubation (Fig. 9) because it is intimately and strongly associated with TDTMA in the montmorillonite interlayers as well as being physically inaccessible to PAH-degrading bacteria (e.g. *Burkholderia*) and their enzymes. By contrast, these bacteria can use free (non-intercalated) Ph as a carbon and energy source (Theng et al. 2001).

Biodegradation can only take place if the sorbed pollutants can be rapidly released from the organoclay complex as is the case with naphthalene (Crocker et al. 1995) and the herbicide fenamiphos (Singh et al. 2003).

3.4 Uptake of Anions

Type II organoclays are also useful for sorbing anions from aqueous solutions as Bors and co-workers (Bors 1990, Bors and Gorny 1992, Bors et al. 2000) have found for iodide and pertechnetate (TcO_4^-) whose radioactive forms are hazardous components of nuclear waste. Sorption efficiency is related to the extent of loading by the organic cation, a prime requirement being the development of positive surface charges or positvely charged regions on the clay mineral. This requirement is usually met when the degree of occupancy by the quaternary ammonium cation exceeds the cation exchange capacity of the smectite (Xu and Boyd 1995, Churchman 2002).

Brixie and Boyd (1994) have evaluated the effectiveness of nine organoclays in reducing the leachability of pentachlorophenol (PCP) from three highly contaminated soils. Although all the organoclays tested were effective in decreasing the concentration of PCP (as the anion) in the leachate, type II organoclays were greatly superior to their type I counterparts. Thus, the addition of 20% dimethyldicocoammonium smectite to the soil reduced leachable PCP levels to below the detection limit (0.2 mg L^{-1}).

3.5 Uptake of Heavy Metal Cations

Organoclays are generally not very effective in taking up heavy metal cations since the interlayer quaternary ammonium cations (QACs) are not readily exchangeable. This limitation, however, may be overcome by intercalating QACs containing an anionic functional group into the smectite the resultant organoclay can serve as an efficient sorbent of both NOCs and heavy metals. For example, Sheng et al. (1999) found that a carboxydecyltriethylammonium (CDTEA)-montmorillonite had a much higher capacity for removing Pb^{2+} from solution than either the sodium- or decyltrimethylammonium (DTMA)-exchanged forms of the clay mineral, while its ability to take up chlorobenzene was comparable to that shown by DTMA-montmorillonite. They suggested that Pb^{2+} interacted with the carboxyl group of CDTEA, while chlorobenzene was

partitioned into the organic phase. Similarly, montmorillonite exchanged with an organic cation containing the thiol (-SH) metal-chelating functional group, such as 2-mercaptoethylammonium, was effective in taking up Hg^{2+} and Pb^{2+} from solution (Celis et al. 2000).

It seems clear that the uptake by organoclays of NOCs and anthropogenic organic pollutants is influenced by many factors related to the nature, properties, and structures of both sorbate and sorbent as well as the composition of the solution phase. As a result, the mechanisms involved in the organoclay-NOC interaction are often difficult to deduce with certainty although some general principles, as outlined above, are beginning to emerge.

4 Some Practical Applications

4.1 Water Treatment

The use of organoclays, especially of type II, for removing oil and grease from water, aromatics from oily liquid wastes, and oil from oil-in-water emulsions has been well documented (Adebajo et al. 2003). Figure 10 shows the effectiveness of an organoclay in taking up a range of mineral oils from water. Organoclays are especially efficient in removing non-polar oils. The results for turpentine indicate that organoclays are far superior to the more expensive activated carbon. An added disadvantage of activated carbon is that its pore space is prone to clogging by the organic contaminants (Alther 2002).

Nevertheless, organoclays (in powder form) are often used in conjunction with activated carbon for the treatment of oily wastewaters, acidic oil well fluids, and boiler feed water in order to reduce overall cost of operation, and prolong the useful life-time of the carbon (Beall 2003). Similarly, Alther (2002) has found that an organoclay/carbon column system is much more effective in taking up petroleum hydrocarbons (from water) than either sorbent alone. Organoclays are also efficient in treating potable water since they can remove dissolved natural organic matter that gives rise to toxic trihalomethanes when the water is chlorinated. Figure 11 compares the efficiency of an organoclay in removing humic acid from a Florida groundwater with that of activated carbon. Besides being superior to activated carbon in its performance, the organoclay column can be regenerated by backwashing with a solution of 1 M NaOH (Beall 2003).

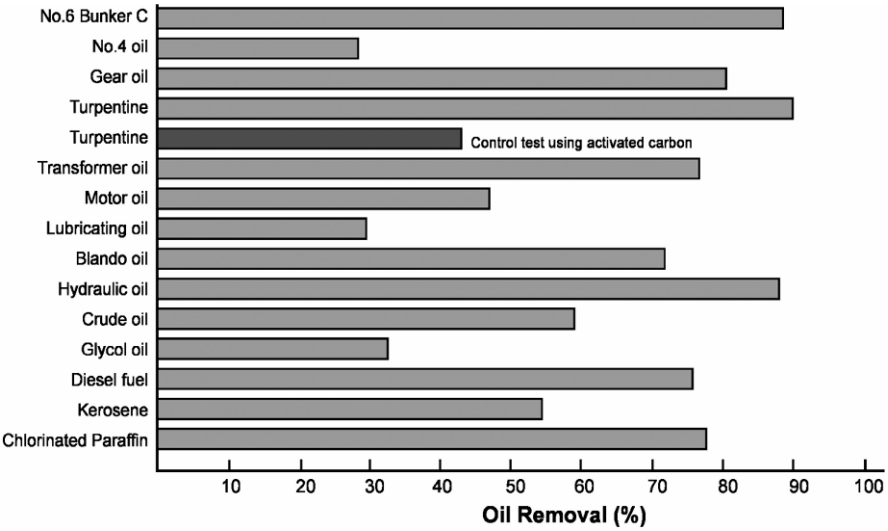

Fig. 10. Diagram showing the efficiency of an organoclay in removing different mineral oils from water, using the 'jar test' or single-point isotherm. After: Alther (2002).

Similarly, Gates and Slade (2001) have proposed using organoclays for the removal of mycrocystin toxins from blue-green algae that accumulate in waterways and water storages, while Cioffi et al. (2001a) have found a benzyldimethyloctadecylammonium-bentonite to be highly efficient in taking up waste organic materials from industrial processes, such as tanning.

4.2 Organoclay Liners and In-Situ Modification

Since conventional clay liners are adversely affected by organic fluids, the use of organoclays as a secondary containment barrier (e.g., for gasoline from underground storage tanks) has attracted much attention. In comparing the swelling properties of a bentonite and an organoclay, Lo and Yang (2001) have found that bentonite shrinks, while the organoclay swells, when immersed in gasoline. More importantly, the hydraulic conductivity of the organoclay is two orders of magnitude lower than that to water. Indeed, the effectiveness of the organoclay in retarding the advective flow of gasoline is comparable to that of a high-density polyethylene membrane (Yang and Lo 2004). Earlier, Smith and Jaffé (1994) simulated the movement of benzene through conventional sand-bentonite liners and liners that had been amended with

hexadecyltrimethylammonium- and benzyltrimethylammonium-bentonite. The maximum benzene breakthrough increased from ~4 years for the conventional liner to ~275 years for the organobentonite-amended material. Using a similar approach, Lo and Mak (1998) found that an organoclay liner could significantly retard the transport of phenolic compounds as compared with a conventional soil liner (composed of 90% silty sand and 10% natural clay mineral).

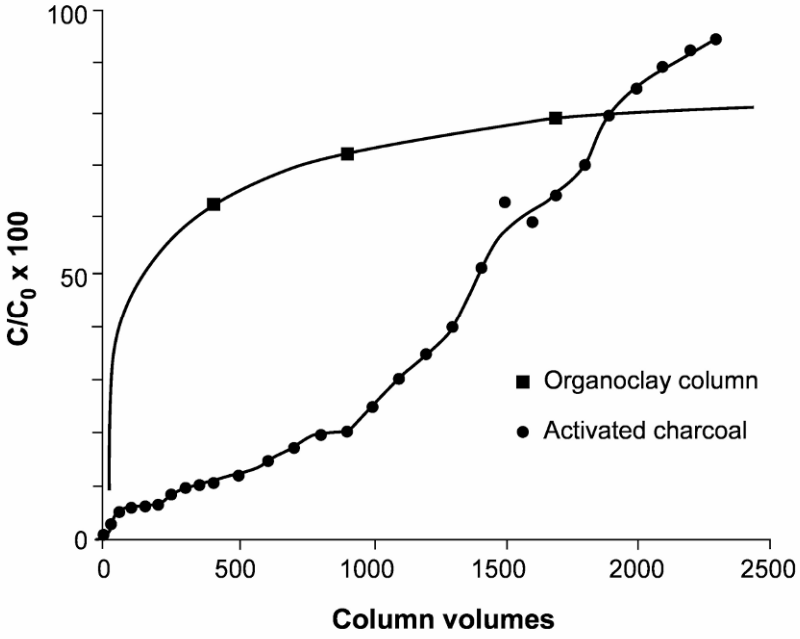

Fig. 11. Diagram showing the efficiency of an organoclay in removing dissolved humic acid from a Florida ground water in comparison with activated carbon. The total organic carbon concentration was 5.6 ppm. See Fig. 8 for the meaning of C/C_0. After: Beall (2003).

The feasibility of forming organoclays in-situ (by replacing the inorganic counterions in subsoils or aquifer materials with long-chain quaternary ammonium ions) to intercept the flow of organic contaminants in subsurface environments, first proposed by Boyd and co-workers in the late 1980s (Boyd et al. 1988, Lee et al. 1989), has continued to attract much interest (Burris and Antworth 1992, Brown and Burris 1996, McBride et al. 1997, Prost and Yaron 2001). An outline of this process is shown in Fig. 12. Here the organoclay is formed by injecting a solution of hexadecyltrimethylammonium (HDTMA) into a subsoil or aquifer. The

resultant organically modified material, when properly positioned, can take up organic contaminants dissolved in plumes from a buried waste. The retained ('immobilised') contaminants can be degraded *in situ* by native or introduced microbes (Lo et al. 1997, Xu et al. 1997). However, if the sorbed pollutants are not bioavailable, recalcitrant, or highly toxic, they may be contained by solidification in cements (Montgomery et al. 1988, Lo 1996, Cioffi et al. 2001b).

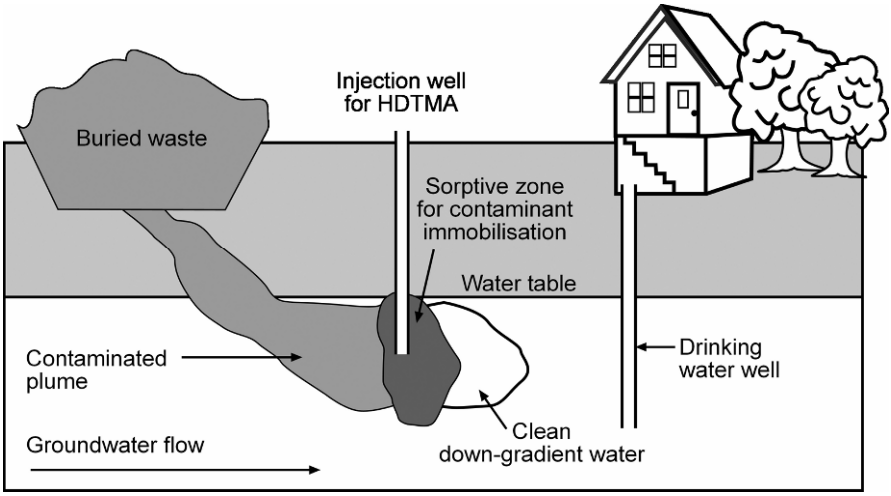

Fig. 12. Scenario for the in-situ modification of subsoils or aquifers for pollution control. The organoclay formed by injecting a solution of long-chain quaternary ammonium cations (e.g. HDTMA) acts as a sorptive zone where organic contaminants dissolved in a plume from buried waste can be immobilised and degraded. After: Xu et al. (1997).

4.3 Pesticide Carriers and Formulations

As might be expected, organoclays are capable of taking up a wide range and variety of non-ionic herbicides and pesticides through solute-surface interactions and partitioning as described earlier (Rodríguez-Gonzalo et al. 1993; Cox et al. 2001; Carrizosa et al. 2004). With ionisable pesticides, such as dicamba (3,6-dichloro-2-methoxy benzoic acid) solution pH is an important factor affecting sorption (Zhao et al. 1996). Since sorption of pesticides retards their leaching in water, and reduce their volatility to air, organoclays and organoclay formulations can usefully serve as carriers of slow-release pesticides (Hermosin et al. 2001, Nir et al. 2006). A novel

approach, proposed by Groisman et al. (2004) is to use a bifunctional organoclay that can act as both sorbent and detoxifying agent of pesticides. In this instance, the organoclay was a Na^+-montmorillonite intercalated with decyldimethyl-2-aminoethyl ammonium, and the pesticides were methyl parathion and tetrachlorvinphos. The aminoethyl ('second') functional group of the quaternary ammonium ion catalyses the hydrolysis of the sorbed organophosphates, leading to their detoxification.

5 Concluding Remarks

Although the theoretical principles behind the interactions of organic cations with clay minerals are reasonably well understood, the practical synthesis of 'tailor-made' organoclays for pollution abatement and containment is not always straight-forward because of the many variables involved. For example, the development of bifunctional organoclays capable of taking up specific pollutants as well as rendering them harmless by chemical conversion and degradation is hampered by their high cost and specificity as do their environmental applications. Similarly, the ability of organoclays in taking up organic contaminants from solution is now well documented but our understanding of the underlying mechanisms at the molecular level is still incomplete, especially with respect to the release and bioavailability of the sorbed contaminants. Nevertheless, this brief survey of the literature would indicate that there is considerable scope for using organically modified clays in cleaning up oil spills, and removing dissolved organic matter and humic substances from water. The stability and low cost of organoclays, relative to such materials as activated carbon, are an added advantage. Likewise, organoclay liners are capable of retarding, and even preventing, the movement of some organic pollutants (e.g., petroleum hydrocarbons) in subsurface environments. In this context, the possibility of producing organoclays in-situ to intercept and contain harmful contaminants from buried waste (landfills) is especially attractive. Organoclays are equally useful as carriers of slow-release pesticides by reducing their leaching and volatilisation. This application has great potential in view of the increased usage of chemicals for pest control and agricultural production in Oceania and elsewhere, and the resultant contamination of soils and waters (Theng et al. 2000).

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7 Microbial Transformation of Organic Chemicals in Natural Environments: Fate of Chemicals and Substantiation of Microbial Involvement through Enrichment Culturing Techniques

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1 Introduction

Environmental organic pollutants, mostly released through industrial processes, application and accidental spills at high quantities, can be partitioned among several physically distinctive compartments of the sediment including clay minerals, organic matter and biologically active biomass. Among these three, biological processes, including both microbial mediated or non-microbial but organic/enzymatic catalyzed, have been recognized for their roles in transformation and, sometimes completely elimination of organic pollutants in the environment (Bollag and Liu 1990; Dilly

et al. 2004). Bioremediation by utilization of selective microbial catalyzed biochemical processes and plants is a very attractive technology in dealing with a wide range of environmental contaminants and *in situ* cleaning up (Atlas 1995; Kuo and Genthner 1996; Jhanson et al. 1999; Whiteley and Bailey 2000; Yu and Gu 2006, 2007a–d; Yu et al. 2007a,b). At the same time, interactions between organic or inorganic pollutants with organic constituents of the sediments have also been actively studied for a better understanding of the fate of chemicals and the chemical processes and mechanisms involved (Wang et al. 1986; Grossart et al. 2003; Montville and Schaffner 2003; Bakker et al. 2004). Among these, an apparent lack of information is about the contributions by biological transformation and inorganic catalyzed process to the overall elimination of pollutants in the environment (Skipper et al. 1967; Yin et al. 2000; Christensen et al. 2002; Gu et al. 2003a,b; Kleinsteuber et al. 2006).

In addition, a large quantities of the literature still show research data on degradation based on comparison between biologically active vs. sterilized system by autoclaving to illustrate and support the biotransformation responsible for elimination of chemicals (Jhanson et al. 1999; Kanaly et al. 2002; Obst et al. 2005). This approach may provide initial information on the fate of chemical pollutants in the simulated environment yielding indication of the biodegradability of the chemical concerned, but further mechanisms of biochemical transformation and/or the identity of the microorganisms involved cannot be revealed from the data obtained. The fundamental information about the biological processes involved and the microorganism acting on the chemicals can only be achieved by further the experimental approach through isolation of the microorganisms and then elucidation of the biochemical pathway (Gu and Berry 1991, 1992; Gu et al. 2005). In this way, fundamental understanding of the chemistry of biotransformation, and both basic microbial physiology and biochemistry can be achieved. In the whole process of investigation of the mechanism, abiotic contribution can be minimized or eliminated completely because the testing system will be depleted with environment constituents derived from the sediment used as an inoculum.

Because of the abiotic process participating in biological transformation and similarly biological process in non-biological process. Fate of environmental pollutants should be investigated with the overall view about the transformation by abiotic and biological processes so that the information obtained can be comprehensive for an in-depth understanding of the pollutants in the environment with minimum bias. Because of these, the purpose of this chapter is to provide a discussion about biodegradation study where abiotic processes also play an important role.

2 Occurrence of Organics in the Environment

Natural organic compounds are part of the food chains for microorganisms and play an important role in the cycling of nutrients including C, N and S (McGill et al. 1981). Natural ecosystem has its own ability in self-regulating, purifying and maintaining the efficient function of various components achieving a balance between various participants. Such cooperation between various organisms and physical components of the ecosystem has been able to sustain the ecosystem for millions of years. However, such balance is disturbed when large quantities of pollutants are being discharged primarily due to their toxicity to the biota and impair the normal function of the selective members of the ecosystem (Mueller and Nielsen 1996; Yu et al. 2005; Rooney et al. 2006; Yu and Gu 2006; Yu et al. 2007a,b). Many examples are available on the effect of chemicals, e.g., polychlorinated biphenols, dioxin, BETX (benzene, ethylbenzene, toluene and xylene), methyl *tert*-butyl ether (MTBE), etc. One obvious fact is that little information is available on the abiotic process contributing to the transformation or elimination of chemicals in the environment even though information indicated that such process may contribute to 30–40% of the parent compound loss when herbicides atrazine, cyanazine and dicamba were incubated with sediment from wetland of Virginia, USA (Berry et al. 1991; Gu et al. 1992), southern, central and northern China (Gu et al. 2003a) and river sediment of Pearl River in southern China (Gu et al. 2003b; Lin et al. 2006). Using radiolabelled atrazine in incubation study conducted in laboratory, 73% of the initial atrazine was degraded and immobilized in the first 21–28 days and at the same time period only 2.2% of the chemical were evolved by microbial respiration (Skipper et al. 1967). Hydrolytical reaction was the key one in transforming atrazine to hydroxyatrazine. Similar results were also obtained by others using different soils (Armstrong et al. 1967).

Environmental organic pollutants may be degraded depending on their toxicity, solubility, distribution constant K_{ow} because physical properties of hydrophobic chemicals may affect the solubility and therefore the amount of organic carbon available in the aqueous phase for microbial assimilation and further metabolism (Schwarzenbach and Westall 1981). Chemicals are subject to volatilization and such loss is not assessed in most of the study except for physical transformation and material balance purposes. Polyaromatic hydrocarbons (PAHs) are known to volatilized during incubation even with capping and more then 40% of the initial chemicals could be found lost (Yin and Gu, unpublished data). When proper control was not included and such

information was interpreted as degradation, incorrect conclusion can be drawn. At the same time, chemicals in such study may be abiotically transformed by the presence of clay materials (Miller and Alexander 1991) and complexed with the organic matter of the sediment (Mortland 1970). Both the clay materials and organic matter in the sediment can contribute to the concentration decrease of a target chemical (McBride 1987; McBride et al. 1988). Such information is largely not being considered by biologists in their investigation.

3 Photo-Degradation

Under natural condition, persistent chemicals are also synthesized by the normal function of the ecosystem, e.g., fluorinated and chlorinated compounds by marine algae. Chlorinated organic compounds are known being produced by marine algae in marine environment (Gschwend et al. 1985), but their environmental concentrations never exceed the levels that would pose significant inhibition to the members of the natural community except for antagonistis purposes. When such chemical are produced for inhibition purpose, their effects are associated with the activity and physiology of the host organisms. The reason for this is that such chemicals are only produced at very low concentration to serve their purpose for deterrence or as repellent. On the other hand, these chemicals are also transformed possibly by organisms and/or abiotic processes at low concentration more efficiently, leaving low residual concentration in the environment. One example for such process contributing to the destruction of chemical in the environment is by naturally produced free radical.

Free radicals can be generated by a number of processes, e.g., thermolysis, photolysis, ionizing radiolysis, redox reactions, the Fenton reaction, chain reactions and mechanical generation. In aqueous systems, free radicals are mostly produced by photochemical processes. Highly reactive species including $^1O_2$, $OH^\bullet$, H_2O_2 and organic peroxides in oxygenated waters react with a range of organic compounds and the surface molecules of microorganisms. For example, in a typical summer, surface waters receive about 1 kW m^{-2} of sunlight, equivalent to about 2 mol photo m^{-2} in the region of 300–500 nm (Hoigné 1990). A significant portion of the photons is actually absorbed by dissolved organic compounds in the surface water and a portion is believed to react with nitrate or nitrite and Fe species (Table 1). However, it is not known to what extent microorganisms are impacted by the redox reaction and, in particular the generated free radical species under natural conditions.

Equally, little is known about the interactions between such active species and the environmental pollutants. Electrochemically generated $^{\bullet}OH^{-}$ radicals are highly effective in killing test strain of *Escherichia coli* and cellular morphology was observed to be deformed and lysed (Diao et al. 2004). Similarly, reactive species of the Fenton reaction also have similar effects on bacterial viability and a large collections of chemicals including MTBE (Xu and Gu 2004; Xu et al. 2004, 2006), a gasoline oxygenate known to be resistant to degradation by microorganisms, can be eliminated. Coupling of the oxidation of *s*-triazine including atrazine, simazine, trietazine, prometon and prometryn with particulate TiO_2 as photocatalyst under simulated solar light achieved degradation of these compounds, but mineralization was not achieved (Pelizzetti et al. 1990). The degradation intermediate cyanuric acid can persist in the environment and poses unexpected impacts on the ecosystem and the components of the living system.

Table 1. Reactants produced by photochemical processes in natural waters

Products	Chemical Formula	Possible generation processes
Singlet oxygen	$^1O_2$	Light absorbing dissolved organic matter (humic acids)
Superoxide anion	O_2^-	Photolysis of Fe^{3+} complexes; deprotonation of HO_2
Hydroperoxyl	HO_2	Protonation of O_2^-
Hydrogen peroxide	H_2O_2	Photolysis of Fe^{3+} complexes, disprotonation of superoxide anion
Ozone	O_3	Uptake from atmosphere
Hydroxyl radical	OH	Photolysis of hydroxo or other Fe^{3+} complexes, of NO_3^-, NO_2^-, photolysis of H_2O_2
Organic peroxy radicals	ROO	Photolysis of dissolved organic material

Modified from Stumm and Morgan (1996).

Such reactions have only been investigated on their role in the processes of ageing and carcinogenesis to a large extent (Dizdaroglu 1991), their effects on environmental contaminants and organisms have not been fully realized. Because of the short time and difficulties in chemical analyses for transitory presence of free radicals, relationship between free radical concentrations and effects on pollutants or natural microorganisms has not been fully established. It is clear that further

investigation on the abiotic catalytic mechanisms for transformation of organic pollutants will provide important information on the assessment of pollutant fate in the environment.

4 Hydrolysis

One of the most common abiotic reaction is hydrolysis because certain chemical bonds, e.g., ester, are very susceptible to chemical hydrolysis in aqueous solution, especially at low pH. High molecular weight chemicals, e.g., polymers, need to be hydrolyzed to smaller molecules before they can be assimilated into the bacterial cells (Gross et al. 1995; Gu et al. 1993a,b,c; 1994; Gu and Mitchell 1995). It should also be reminded that esterase is also an important group of enzymes commonly detected in a wide range of microorganisms including fungi and bacteria for transformation. When catalyzed by microorganisms, the reaction seems to have an unexpected high diversity of esterase in the environment as recently observed on de-esterification of phthalate ester isomers (Wang et al. 2003a; b; Gu et al. 2002, 2005; Li et al. 2005a,b; Li and Gu 2006; Wang et al. 2006; Li and Gu 2007). As shown in these investigations using dimethylphthalate esters, removal of the chemically identical two ester bonded methyl groups are carried out frequently by different species of bacteria, suggesting the selectivity of the microorganims involved for the specific chemical bond catalyzed.

Many hydrophobic chemicals including polyaromatic hydrocarbons (PAHs) are less soluble in water and are initially activated by oxygenase or P450 system in which oxygen from O_2 can be introduced to the parent compounds making them more soluble in the initial phase of transformation under aerobic conditions (Bollag and Liu 1990). When molecular O_2 is not available, such as under methanogenic and sulfate-reducing conditions, initial attack of the chemical can be initiated through a range of reactions including hydrogenation by phototrophic micoorganims (Berry et al. 1987), hydroxylation in methanogenic consortia of bacteria acting on indole and 3-methylindole (Gu and Berry 1991, 1992). Further transformation under anaerobic conditions involves addition of oxygen from H_2O molecule to the substrate. However, it should also be pointed out that abiotic transformation may take place catalyzed by surfaces of clay minerals to achieve hydrolysis and such degradation may have a positive effect on the overall degradation of the chemical. The reason is that majority of the environmental samples we are dealing with contain a significant fraction of sediment materials and therefore their effects on transformation of chemicals is apparent.

Clay minerals are structure of aluminum- and silicate-oxides. Such structures have free bond and vacancies on selective site of the mineral structure allowing them to participate in both physical adsorption and chemical reactions modifying the available concentration of the chemicals in the environment.

5 Biochemical Processes

Apart from hydrolysis mentioned above, other specific biochemical reactions can also participate, especially those catalyzed by microorganisms under aerobic and anaerobic conditions, in the degradation of a wide range of chemicals. One example is the esterase catalyzed reaction in the initial hydrolysis of ester compounds as demonstrated using dimethylphtalate ester isomers, including dimethylphthalate, dimethylterephthalate, and dimethylisophthalate recently (Gu et al. 2002; Wang et al. 2003a,b, 2004; Gu et al. 2004, 2005; Li et al. 2005a,b; Li and Gu 2006; Wang and Gu 2006; Wang et al. 2006; Xu et al. 2005a,b). A diverse group of microorganisms has been confirmed to be involved in the degradation of this class of chemicals from several selective environments including activated sludge, mangrove sediment and deep-ocean sediment, and their specific involvement has only being partially substantiated recently using bacteria isolated (Wang et al. 2003a, b; Gu et al. 2004, 2005; Li et al. 2005a,b; Wang and Gu 2006; Li and Gu 2007). Interestingly, most of the microorganisms are commonly found with previously established physiological function in the environment (Table 2), but their biochemical capability in degrading this class of ester isomers are quite variable, especially on the initial hydrolytic reaction. Only a few of these isomers are being mineralized; in most cases these esters require the collaboration between two specific microorganisms to achieve complete mineraliztion of the isomer involved. The initial ester hydrolysis is widely acknowledged, but the diesters in most of the time require two different microorganisms to carry out this hydrolytic step. Such high specificity between a chemical and the microorganism is rare, indicting the stereo-structural effects and chemical reaction carried out by microorganisms in metabolizing environmental pollutants.

Two processes of microbial degradation must be emphasized in our understanding the fate of chemicals in the environment, metabolism via mineralization or co-metabolism. The former is specifically for process carried by bacterial and support the growth of the microorganisms while the latter one involves the presence of a second source of carbon and energy in which the microorganisms actually use these for growth, but also

transform the pollutants. Because of this, recalcitrant chemicals can also be transformed by microorganisms in the environment. However, we recently observed that exchange between methyl group on phthalate aromatic moiety and the ethyl group in solution phase as ethanol can facilitate the further accelerated degradation of monoethylphthalate, which otherwise would be degraded much slowly, as shown below (Fig. 1) (Li and Gu 2006). This process can be carried out in industrial setting for food processing at high temperature and pressure only, but our experimental conditions were carried out at ambient conditions. This observation indicated that the involved enzyme requires further understanding the biochemical reaction mechanism and may have great potential for application.

5.1 Microbial Transformation

Microorganisms are predominant in the environment and they may directly be involved in degradation and transformation of a wide range of chemicals (Alexander 1994). The chemicals include agricultural pesticides and herbicides (Gu et al. 2003a,b; Lin et al. 2006), industrial solvents, and other chemicals with specific purposes of applications (Yin et al. 2005, 2006). For degradation of specific chemicals, microorganisms can be isolated from various sources for capability of degradation under selective conditions because microorganisms have their preferred niches and biochemical capabilities. In most of these studies, the first step in planning an investigation for a chemical is the experimental conditions in which the experiment will be set up. The degradation investigations are mostly conducted under aerobic conditions for convenience of maintenance and also for the large number of such microorganisms isolated from the environment (Gu and Berry 1991, 1992; Gu et al. 1993a,b,c, 1994; Middeldorp et al. 1997; Adrian et al. 1998; Boonchan et al. 2000; Kanaly et al. 2000; Carvalho et al. 2002; Fan et al. 2004). Another hidden reason is the complexity of anaerobic experiment and the very slow growth of bacteria (Haggblom and Young 1995; Kuo and Genthner 1996; Lepine et al. 1996; Ficker et al. 1999; Pancost et al. 2000; Koizumi et al. 2002; Arias and Tebo 2003; Callaghan et al. 2006; Cheung and Gu 2007), which deters many investigators from using such tedious technical approaches. Under different environmental.

Table 2. Microorganisms capable of degrading phthalate esters completely or partially

Substrates	Microorganisms	Degradation	References
DMP	*Pseudomonas fluorescens, Pseudomonas aureofaciens* and *Sphingomonas paucimobilis*	Complete	Wang et al. (2003a)
	Xanthomonas maltophilia and *Sphingomonas paucimobilis*	Complete	Wang et al. (2003b)
	Pseudomonas fluorenscens FS1	Complete	Zeng et al. (2004)
	Micrococcus sp. strain 12B	Complete	Keyser et al. (1976)
	Bacillus sp.	Complete	Niazi et al. (2001)
	Arthrobacter sp.	Partial	Vega and Bastide (2003)
DMTP	*Aspergillus niger* (fungus)	Complete	Ganji et al. (1995)
	Sphingomonas paucimobilis	Complete	Li et al. (2005a)
	Sclerotium rolfsii (fungus)	Partial	Sivamurthy et al. (1991)
	Comamonas acidovorans D-4	Complete	Patel et al. (1998)
	Pasteurella multocida Sa	Complete	Li et al. (2005b); Li and Gu (2006)
	Sphingomonas yanoikuyae DOS01	Partial	Wang and Gu (2006)
	Viarovorax paradoxus T4	Complete	Wang and Gu (2006)
DMIP	*Klebsiella oxytoca*	Partial	Li and Gu (2004)
	Rhodococcus erythropolis 5D	Complete	Aleshchenkova et al. (1997)
	Rhodococcus rubber 1B	Complete	Aleshchenkova et al. (1997)
	Viarovorax paradoxus T4	Complete	Wang and Gu (2006)
DIBP	*Pseudomonas. fluoresences* FS1	Complete	Zeng et al. (2004)
	Micrococcus sp. strain 12B	Complete	Eaton and Ribbons (1982)

Table 2. (Continued)

Substrates	Microorganisms	Degradation	References
DEHP	*Acinetobacter* sp.	Complete	Hashizume et al. (2002)
DBP	*Acinetobacter* sp.	Complete	Hashizume et al. (2002)

DMP, dimethylphthalate; DMTP, dimethylterephthalate; DMIP, dimethylisophthalate; DIBP, dibutylphthalate; DEHP, diethylhexylphthalate; DBP, dibutylphthalate

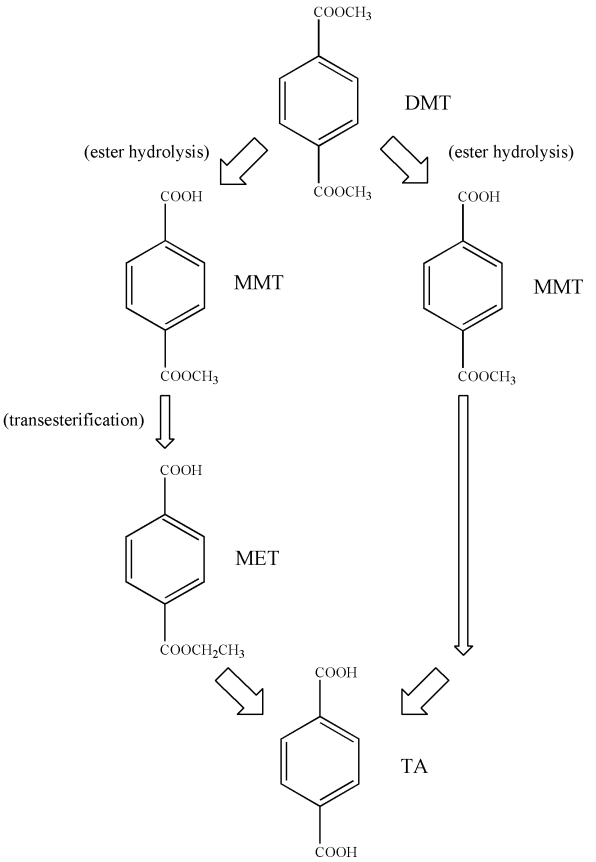

Fig. 1. Biodegradation of dimethyl terephthalate (DMT) by *Pasteurella multocida* Sa in the presence of ethanol. Ester hydrolysis of DMT involves a transesterification of mono-methyl terephthalate (MMT), and the formation of mono-ethyl terephthalate (MET).

conditions, the organic pollutants for such testing are often used as the sole source of electron and carbon for the microorganisms for a large number of such study, but proper electron acceptor need to be provided for oxygen-limited conditions. Oxygen is the preferred electron acceptor under aerobic conditions; NO_3^- when oxygen becomes limited, and SO_4^{2-} and CO_2 when sulfate-reducing or methanogenic conditions are prevalent. Simulation of strictly anaerobic conditions is a big challenge due to the stringent requirement for elimination of molecular O_2 in the experimental system, but also for the very slow growth of the microorganisms. However, when chemical can be degraded at a reasonable rate, corresponding products will be observed to increase over time providing evidence for mineralization of chemicals.

Substantiation of pollutant degradation by microbial process can be carried out using enrichment culturing techniques in which the initial microcosm showing chemical disappearance over time can be used as an inoculum for establishment of a new microcosm containing freshly prepared medium and the target chemical (de Souza et al. 1998; Willardson et al. 1998; de Souza et al. 2000; El-Fantroussi 2000; Wu et al. 2000; Entcheva et al. 2001; Dejonghe et al. 2003; Glaeser and Overmann 2003; Sliwinski and Goodman 2004; Kleinsteuber et al. 2006). If further degradation can be observed in the enrichment transfer culture, further more transfers can be carried out. If 1 in 100 dilution is used in the process, the organic chemical of the sediment or environmental samples can be significantly eliminated after 4 such transfers because only 0.00001% of the initial carbon is present in the 4th enrichment culture and in such system the organic pollutant is the sole source of carbon and energy if they are actually metabolized. Such process has been successfully utilized in enrich microbial consortium responsible for degradation of herbicide cyanazine (Gu et al. 2003a,b) and dicamba (Gu et al. 2003a,b), indole and 3-methylindole (Gu and Berry 1991, 1992; Gu et al. 2001). One difficulty in the enrichment process is that not all microorganisms can derive their cellular constituents from the utilization of organic pollutants and some may require the supplement of vitamins and growth cofactors, but the information for such requirement can only be established on case-by-case basis, which may make such investigation much more time and energy consuming. Indicative observation of disappearance of chemicals in enrichment cultures can be substantiated over time as the number of enrichment increases. This practice can effectively eliminate the questionable data observed as environmental samples are more likely used in the initial phase of such similar investigation.

Stable enrichment culture provides the fundamental basis for further characterization of the microorganisms involved and the biochemical pathway of degradation for the target pollutants. In such system, reproducible data can be obtained reliably without much ambiguity. Traditionally, environmental microbiologists believe that at least one microorganism exists for each pollutants in the environment (Alexander 1994). The infallibility theory by Martin Alexander may still occupy the fundamental thinking about the environmental degradation of pollutants. However, more the more evidences also reveal new information about the metabolic network operated by the environmental microorganisms. Though the number of bacteria that have been in pure cultures and described are high since the availability of gene sequencing technique, new biochemical processes have not been reported in similar pace (Wackett and Hershberger 2001), indicating possibly lack of new biochemical information about the diversity of the biological ecosystem.

5.2 Elucidation of Mechanisms Through Enrichment

Biochemical process of degradation is best elucidated with either pure culture of microorganisms or purified enzymes from the relevant organisms. Because of this, it is a constant struggle for environmental microbiologists to isolate new bacteria or bacteria capable of utilizing a chemical at higher concentration. Conventional approach prefers the enrichment of bacteria with increasing concentrations of the target chemical for easy maintenance and fast growth. During the process, the cells capable of degradation can increase their number through biochemically degrading the chemical while those that are not as competitive as the former will be eliminated during the dilution enrichment transfer process as described above. Because the transfer process involve inoculation of freshly prepared culture medium with a fractions of the active culture previously established (Fig. 2). Assuming a 1% culture taken for inoculation, the first enrichment transfer receive 1% of the initial culture materials including the environment materials, specifically sediment and carbon. Further transfers will results in reducing the original culture to 1%, 0.01%, 0.0001% after 2, 3, and 4 times of series enrichment transfers, respectively. At the same time, the active microorganism will increase in their number and those non-active cells would be almost completely eliminated after 5–6 enrichment transfers if there is at least one microorganisms capable of degradation. At the same time, this enrichment process eliminates the residual organic C and sediment materials from the sediment, which magnify the signal of biodegradation by microorganisms that utilize the chemical as the sole source of C and energy.

Fig. 2. Scanning electron micrograph showing a natural microbial biofilm developed on surface of immobilized surface when dimethylphthalate was used as the sole source of carbon and energy after dehydration and critical-point dried and coating with palladium and gold (unpublished results).

This process has been instrument in the isolation of a number of pollutant-degrading microbial consortia for industrial chemicals (Wang et al. 2003a,b; 2004), agricultural ones (Gu et al. 2003a,b; Lin et al. 2006) and others including metals (Cheung and Gu 2005; Cheung et al. 2006).

It should also be noted that not all chemicals can be degraded by one single specie of bacteria. One example is the biochemical collaboration between different species in utilization of one organic compound as recently demonstrated on phthalate ester isomers (Wang et al. 2003a,b; Gu et al. 2004, 2005; Li et al. 2005a,b; Li and Gu 2006, 2007). This specific case illustrates the complexity of microbial community in term of substrate C utilization (Fig. 3). However, such biochemical cooperation also offers advantage for the existence of a community, in particular for the common survival, because initially each microorganism receive only one C from hydrolysis of the methyl group of the ester bond and they can compete for the remaining C after the hydrolysis of both ester bonds. Because of this mechanisms, there is a common opportunity for different members in the community to co-exist in the same environment.

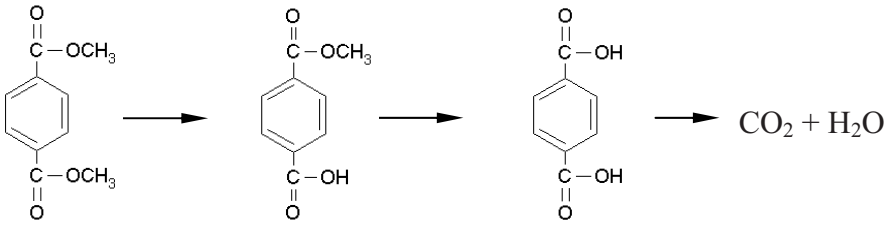

Fig. 3. A proposed biochemical pathway for aerobic degradation of dimethyl terephthalate (DMT) by *Pasteurella multocida* Sa isolated from mangrove sediment (Li and Gu 2006).

6 Interactions Between Living and Non-Living Components of the Ecosystem

Physical environment play a very important role in preserving biota by the surfaces and also supplying the available carbon through desorption and other mechanism affecting degradation processes. Sediments are largely consisted of minerals and organic matter and living biomass depending on the physical and chemical conditions. However, during microbial transformation study, emphasis has been mostly placed on the chemical transformation and the possible involvement of microorganisms by examining and comparing the chemical concentrations between biologically active and the sterile control. Rarely has attention been given to the interactions between the inorganic constituents and the microorganisms, and possible effect of the former on the latter. Actually, microorganisms may benefits from the surfaces provided by the clay minerals and possibly the catalysis by clay minerals in transformation of organic pollutants.

N-heterocyclic aromatic indole can be mineralized by methanogenic enrichment culture of bacteria from municipal sewage sludge to methane as shown in previous study (Gu and Berry 1991, 1992). Degradation of indole was observed to be affect by the presence of montmorillonite at two concentrations 2 and 4 mg/ml (Fig. 4) by the stable indole-degrading methanogenic consortium capable of minertalizing indole through oxindole and isatin as reported earlier (Gu and Berry 1991, 1992). When the anaerobic microcosms were prepared with addition of the 2:1 clay mineral montmorillonite, an initial delay in degradation was observe as the amount of montmorillonite increased, suggesting that surface of clay mineral may adsorb the indole in aqueous solution and/or the degradative microorganisms so that the activities of microorganisms were affected.

Because of these, mineralization product methane also showed corresponding delay as the amount of montmorillonite increased in the culturing microcosms. However, very little information is available in this area as to the mechanisms involved and further research in this area will allow better understanding and assessment of interactions between microorganisms and organic compounds in the environment (Lünsdorf et al. 2000).

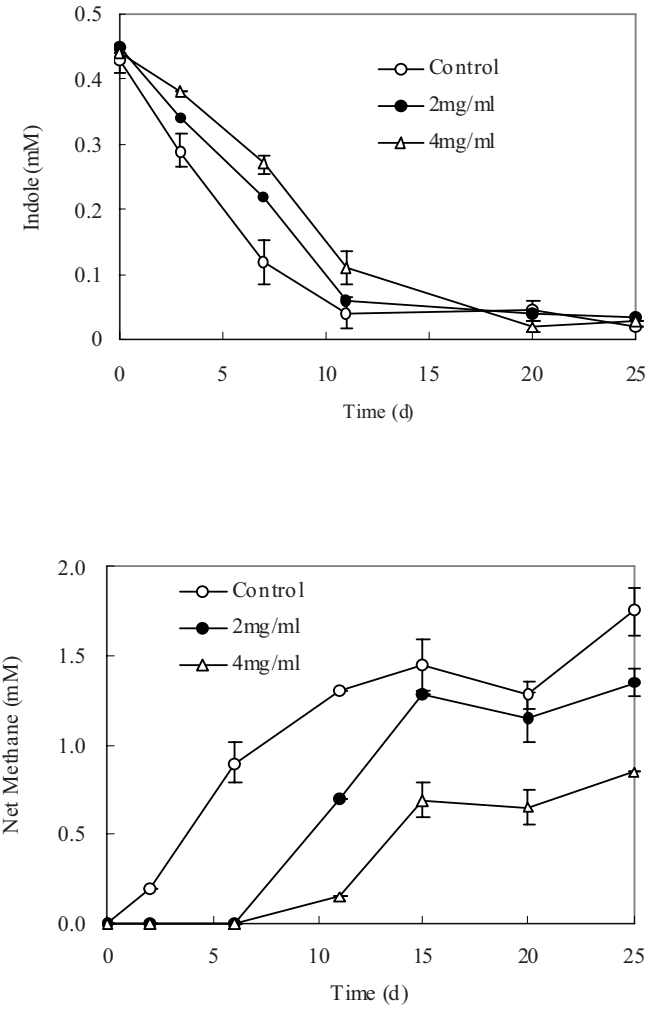

Fig. 4. Effect of montmorillonite on degradation of indole by an indole-degrading methanogenic consortium incubated under strictly anaerobic conditions (unpublished results).

7 Ecotoxicological Assessment

Most ecological risk analyses, using information based on concentrations of environmental chemicals analytically detected and also the toxicological data of the chemicals on selective model animals in laboratory system, make prediction of the long-term exposure by extrapolation by applying various mathematical techniques (Yu and Gu 2006 a–d). Such approach has a major drawback in that the effective concentration of the chemical in laboratory conditions that can be exposed to organisms are not the same as those in ecosystem or complex system, e.g., sediment. Knowing the concentration by analytical analysis does not imply accurately reflection of the effects of physical matrices on chemicals that we are dealing with imposing effect on the actual concentration of the chemical available to organisms. Because of this, a better understanding of the physical environment and interactions between physical and biological components will facilitate our better understanding of environmental pollutants in the environment. Such information will improve our prediction because more realistic information about toxic chemical concentration can be obtained.

Bioremediation in situ is much more complicated than the system used in the laboratory controlled condition. Microorganisms in natural community interact with each other and also with other organisms and plants; such interactions are more complicated than the scientific technique can delineate simply. Because of this, more systematic approaches are needed to reveal the connectivity between these biological factors and nonbiological factors to understand the underlying processes.

8 Conclusion

Fate of organic pollutants should be assessed with comprehensive consideration of all factors contributing to the decrease of parent chemical concentration extractable from the system. Both abiotic and biological transformation may play significant role in the change of chemical concentration. When biological degradation is the ultimate goal of the investigation, enrichment culture can be used to both magnify the biological signal and also obtain the microorganisms for further investigation. With the pure culture of bacteria, mechanism of transformation can be further substantiated in laboratory study. However, ecological risk assessment requires that a more accurate quantification of pollutants concentration should be achieved through better understanding of the physical environments

and interaction between minerals and organic matter with the target pollutants. Without this critical step, our prediction will be very limited.

Acknowledgments

Preparation of this manuscript was supported in part by The University of Hong Kong. I would like to thank Zhenye Zhao help in preparation of Fig. 4.

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Part II
Applications

8 Soil Organic Matter Quality and the Size and Activity of the Microbial Biomass: Their Significance to the Quality of Agricultural Soils

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1 Introduction

Concerns regarding progressive soil degradation and the long-term sustainability of current agricultural practices have lead to the study and assessment of soil quality (Karlen et al. 1997). Soil organic matter is an extremely important attribute of soil quality since it profoundly influences

the physical, chemical and biological properties and processes of soils. It is a source of energy and nutrients for soil biota, a plant nutrient (N, S and P via mineralization) source, it contributes to the charge characteristics of soils, it has the ability to complex with multivalent ions and organic compounds and it affects aggregate stability, trafficability, water retention and hydraulic properties. As a result, soil organic matter content and quality are now recognized as key factors in the evaluation of the sustainability of soil management practices (Gregorich et al. 1994, 1997a).

Soil organic matter is a heterogeneous mixture of materials ranging from fresh plant and microbial residues to relatively inert humic compounds. It is often difficult to detect changes in total organic matter in response to alterations in soil management because of the generally high background levels and also natural soil variability (Haynes and Beare 1996). This has led to research into identification of labile pools of organic matter (which make up a relatively small proportion of the total pool) that are more sensitive to changes in soil management or environmental conditions than total soil organic matter content. Examples of such pools include C and N held in the microbial biomass and in particulate organic matter and in water-soluble, easily-extractable and potentially mineralizable fractions (Haynes 2005).

The microbial biomass is the living component of soil organic matter and it typically comprises 1–5% of total organic matter content (Sparling 1997). The microbial biomass plays important roles in the soil since it is both a source and sink for C, N, S and P, an agent for decomposition of litter and formation and degradation of humic material and it has important roles in aggregate formation and degradation (Alexander 1977; Haynes and Beare 1996; Sparling 1997). Because of its high turnover rate, microbial biomass C content can respond rapidly to changes in soil management practice (Gregorich et al. 1997a). Measurement of the size of the microbial biomass gives no indication of microbial activity. The most commonly used index of microbial activity is the measurement of CO_2 evolution from soils (soil respiration rate).

The purpose of this paper is to review the significance of soil organic matter fractions, and the size and activity of the soil microbial community to the function of agricultural soils and investigate how land use and soil management affect these parameters.

2 Total Soil Organic Matter

2.1 Nature of the Pool

Soil organic matter content is generally measured as organic C or total N content. Soil organic matter has long been suggested as the single most important indicator of soil productivity (Allison 1973; Campbell 1978). The main chemical properties affected by soil organic matter are charge characteristics, cation exchange capacity, buffering capacity, formation of soluble and insoluble complexes with metals and interactions with xenobiotics such as pesticides. Key physical properties that are influenced include aggregate formation and stabilization, water retention, resistance and resilience to compaction and thermal properties. The most important biological properties of organic matter are its role as a reservoir of metabolizable energy for microbial and faunal activity, its effect in stabilizing enzyme activity and its value as a source of plant-available N, S and P via mineralization.

Soil organic matter is composed of two major pools; a labile and a stabilized fraction. This is a convenient division although, in fact, soil organic matter includes a continuum of materials ranging from highly decomposable to very recalcitrant. The labile fraction consists mainly of material in transition between fresh plant residues and stabilized organic matter. Much of it is plant and microbial tissue in various stages of decomposition. It is generally considered to have a short turnover time (less than ten years). Pools of organic matter that have been identified as part of the labile fraction include particulate organic matter, soluble C, potentially-mineralizable C, that extractable with various reagents, microbial biomass C and enzymes present in soluble and sorbed forms. Each of these pools defines an aspect of the labile fraction and their significance is discussed in the following sections of this review.

Stabilized organic matter consists of materials that are highly resistant to microbial decomposition because of their chemical structure and/or association with soil minerals. It consists mainly of humic substances which are complex systems of high molecular weight organic molecules made up of phenolic polymers produced from the products of biological degradation of plant and animal residues and the synthetic activity of microorganisms (Stevenson 1994; Baldock and Nelson 2000). Humic substances make up 70–80% of the soil organic matter content of most mineral soils. Since humic substances make up the bulk of soil organic matter content, changes in total organic matter (organic C and total N content) reflect principally changes in the amounts of humic material present.

Based on the physical state, soil organic matter can be divided into light and heavy fractionswith a density of < 1.6 g cm^{-3} and 1.6–2 g cm^{-3}, respectively (von Lützow et al. 2007). Light fraction is commonly referred to a plant-like and less stable fraction with high C concentration. It accounts for 2–17% of the total SOC in surface soils and represents an intermediate pool between undecomposed residues and humified SOM. Light fraction is considered to be the driving force in soil respiration. The importance of light fraction in the formation and stabilization of soil structure is widely recognized. Heavy fraction is a more stable and high density organo-mineral fraction with lower C concentrations. It contains more processed SOM and can be a major sink for C storage in soil because it has less mineralizable C (Tan et al. 2007).

2.2 Effects of Agricultural Activity

In agricultural soils, changes in soil management practice affect soil organic matter content by (i) altering the annual input of organic matter from above- and below-ground plant litter and (ii) by altering the rate at which the decomposer community degrades organic matter and releases organic C to the atmosphere as CO_2. Under any particular long-term soil management practice, soil organic matter content reaches a steady-state level where organic matter accumulation is balanced by losses as CO_2.

Long - term tillage management can significantly change the characteristics of both physical and chemical fractions of SOM (Ding et al. 2002). Long-term field experiments at two European countries revealed that the organic C concentrations were significantly higher in the farmyard manure plots compared to the NPK amendments or control (Böhme et al. 2005). The most obvious effect of agricultural practice occurs when soil under native vegetation is converted to arable agriculture. Typically, organic matter levels decline rapidly in the first 10–20 years and then stabilize at a new equilibrium level after 30–100 years (Haynes and Beare 1996; Paustian et al. 1997; Fenton et al. 1999). A number of factors contribute to the losses of organic matter including: (i) a much lower allocation of carbonaceous residues to the soil (due to the relatively wide spacing of crop plants, removal of harvested products and burning and removal of crop residues, (ii) tillage-induced aggregate disruption and exposure of physically protected organic material to microbial action thus hastening decomposition rates, (iii) more favourable conditions for decomposition (e.g. tillage-induced aeration, irrigation, fertilizer and lime additions) and (iv) greater losses of surface soil by wind and water.

Factors that increase organic matter inputs, and thus tend to increase soil organic matter content under arable agriculture include: (i) a decreasing proportion of fallow in rotation, (ii) increase in the proportion of cereal compared to root crops, (iii) an increasing proportion of perrenial crops (forage legumes and grasses) in rotation, (iv) return of crop residues rather than burning or removal, (v) fertilizer and irrigation additions which promote increased yields and thus greater organic matter returns and (vi) additions of organic manures or other organic wastes (Johnston 1986; Janzen et al. 1997, 1998a,b; Paustian et al. 1997; Fenton et al. 1999; Yang et al. 2007). For example, in a long-term crop experiment with different crops near Linz, Austria, Ros et al. (2006) found that compost treatment increased soil organic carbon at all depths from 11 g kg^{-1} for control soil (without fertilization) to 16.7 g kg^{-1} for the case of sewage sludge compost. A 10-year experiment of legume cover crop incorporation with rainfed Alfisols in southern India showed that biomass incorporation improved mean soil organic carbon content by 24% over fallow (Venkateswarlu et al. 2007). It was suggested by Leifeld and Kögel-Knabner (2005) that the most sensitive fraction to land-use was SOM in the fraction >20 μm not released after sequential wet sieving and ultrasonic dispersion.

The most common way of attempting to reduce the rate of organic matter decomposition is to create less tillage-induced disturbance to the soil by conversion to minimum or zero tillage. This characteristically results in an accumulation of organic matter in the surface few cm (Blevins et al. 1983; Horne et al. 1992) and conservation of organic matter in the soil profile where fields have been converted from native vegetation and soil organic matter is initially relatively high (Dick 1983; Francis and Knight 1993). However, where substantial amounts of organic matter have been lost, through repeated conventional tillage, conversion to zero tillage usually has little effect on total organic matter in the soil profile (Powlson and Jenkinson 1981; Francis and Knight 1993).

In Fig. 1, the effects of various long-term land uses on soil organic C content are shown. A highly productive, fertilized, irrigated kikuyu pasture resulted in a substantial accumulation of organic C compared to undisturbed native grassland. Commercial forestry under *Eucalyptus* (gum) or *Pinus* (pine) forest resulted in similar or greater organic C content than native grassland. This reflects the large litter inputs that occur to the soil under commercial exotic forests. The arable crops (maize under conventional tillage and sugarcane) resulted in a loss of soil organic C. Annual ryegrass pasture resulted in a similar organic C content to native grassland since the

greater dry matter production (and organic matter inputs) are balanced by greater organic matter decomposition induced by annual tillage.

Because of the relatively large quantity of background organic matter already present, changes in organic status caused by changes in soil management practice are usually difficult to detect in the short-term (i.e. < 5 yr) and are usually demonstrated in long-term (e. g. > 25 yr) field experiments (Johnston 1986; Campbell et al. 1997). Indeed, the recalcitrant humic fraction, that makes up the bulk of soil organic matter and has turnover rates measured in thousands of years, is only slowly effected by changes in soil management. By contrast, labile fractions of organic matter have a much greater turnover time and change much more rapidly in response to management-induced changes in organic matter inputs or losses (Gregorich et al. 1994; Janzen et al. 1998a).

3 Labile Organic Matter

3.1 Nature of Pools

3.1.1 Particulate Organic Matter (POM)

Particulate organic matter is transitory pool between fresh plant residues and humified organic matter (Gregorich and Janzen 1996). Often the term light fraction is used as a synonym for POM (von Lützow et al. 2007). While POM and LF are similar, they are not equivalent fraction and have different C-, N-, *O*-alkyl contents (Gregorich et al. 2006).

Particulate organic matter is comprised primarily of plant debris with a recognizable cellular structure but microscopic examination has revealed it also contains fungal hyphae, spores, seeds, faunal skeletons and charcoal (Spycher et al. 1983; Skjemstad et al. 1990). It contains the portion of soil microflora involved in decomposition of residues as well as some humified plant material (Ladd et al. 1977; Baldock et al. 1992). It is thought that particulate organic matter can exist in two major forms; (a) that which is free without significant association with mineral particles (free POM) and (b) occluded forms that are buried within soil aggregates and/or strongly associated with mineral particles (POM occluded in soil aggregates) (Besnard et al. 1996; Gregorich et al. 1997b).

The two forms of POM can be obtained by using density fractionation in combination with ultrasonic dispersion. The chemical composition of both fractions is quite different, with the occluded POM having lower

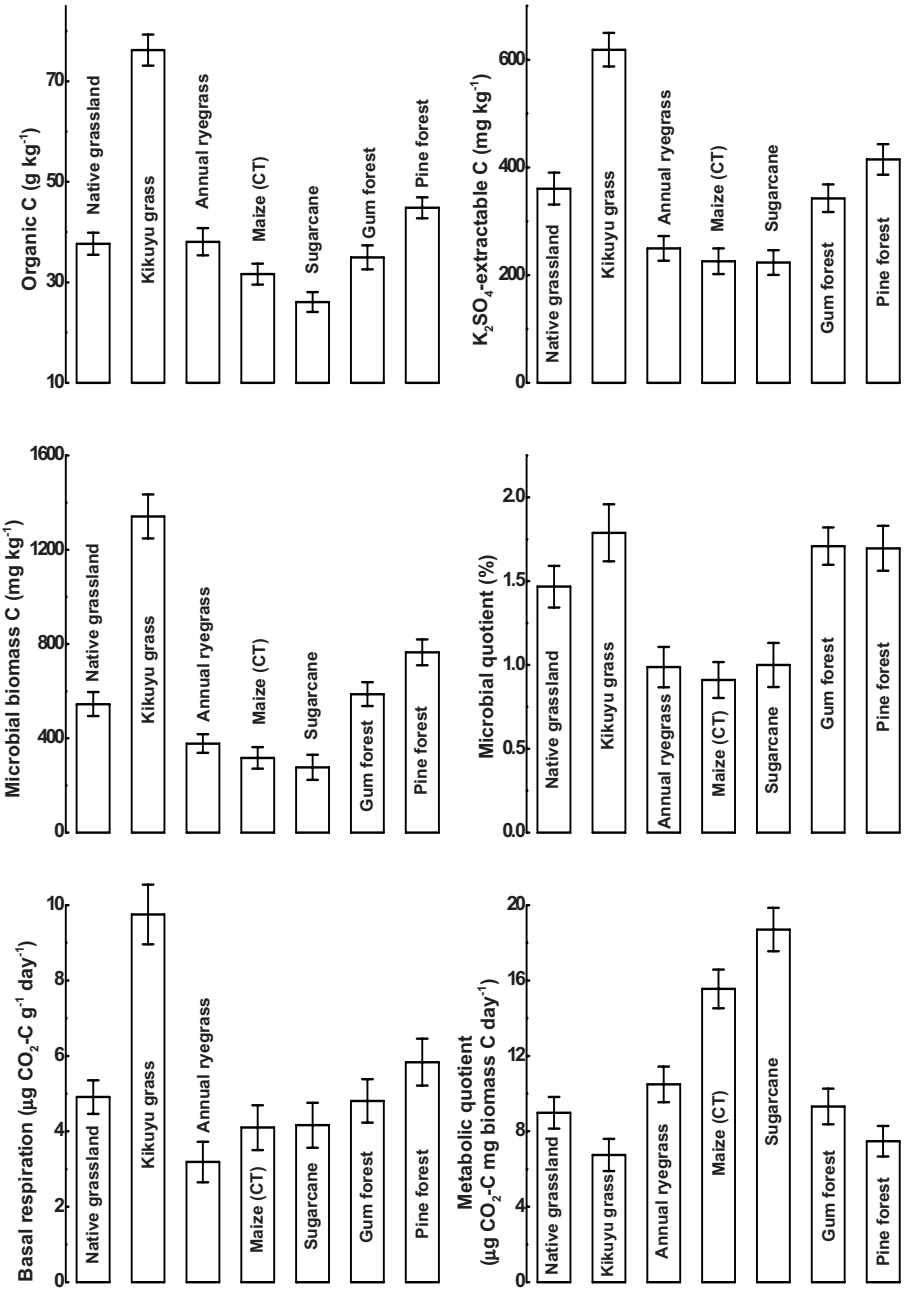

Fig. 1. Effects of long-term land use on organic C, extractable C, microbial biomass C, microbial quotient, basal respiration and the metabolic quotient of soils (0–10 cm). *Bars* are ± S.E. (From Haynes, unpublished, 2005).

amounts of O-alkyl C and higher amounts of aryl C and alkyl C than the free POM (Kölbl et al. 2005). With increasing clay content the amount of organic carbon stored in the occluded POM fraction increased considerably, whereas the amounts of free POM were not related to the soil clay content. Increasing proportions of *O*-alkyl C and decreasing proportions of aryl C were found for both POM fractions with increasing soil clay contents. The occluded POM fraction showed a higher degree of degradation as indicated by lower amounts in *O*-alkyl carbon. A lower degree of POM degradation was associated with higher clay contents. Higher soil clay contents promoted the conservation of POM with a low degree of alteration (Kölbl and Kögel-Knabner 2004). Drying and wetting strongly affected the amounts of POM incorporated into aggregates (intra-aggregate POM, iPOM). More coarse iPOM is decomposed into fine iPOM in macroaggregates not exposed to dry-wet cycles due to a slower macroaggregate turnover (Denef et al. 2001).

Particulate organic matter contributes to soil function in a number of ways. It is the major pathway through which C and nutrients are returned to the soil (as above-ground plant residues and from root turnover). It is the major precursor for formation of other forms of organic matter which are released and/or synthesized during its decomposition. It is a major source of cellular C, energy and nutrients for the heterotrophic soil microflora and indogeic (soil-feeding) soil fauna including many earthworms and termites. Studies have also shown that stable soil macroaggregates tend to have cores of particulate organic matter and that aggregates form around particles of decaying plant residues (Golchin et al. 1994, 1998).

Seasonal fluctuations in particulate organic matter have been recorded by a number of workers (Conti et al. 1992; Boone 1994; Campbell et al. 1999 a,b). These can usually be related to the timing of organic matter inputs (e.g. return of crop residues) and their seasonal pattern of decomposition (Campbell et al. 1999 a,b). Boone (1994) concluded that the seasonality of organic matter inputs to the soil was the main factor affecting the amounts of particulate organic matter extracted from soils under maize. Such seasonal variability needs to be considered when interpreting particulate organic matter data and for comparison, soils should be sampled at the same time each year.

3.1.2 Dissolved Organic Matter (DOM)

Dissolved organic matter can be thought of, for simplicity, as soluble organic matter present in soil solution. However, although some workers have measured dissolved organic matter in soil leachates or extracted soil solutions, many use the extract from a 1:2 w/v soil/water extraction

(Burford and Bremner 1975). A number of salt-extractable fractions have also been used as measures of dissolved organic matter. Salt extracts are used mainly for ease of extraction since they cause flocculation of soil colloids present in aqueous extracts. The most common salts used are 0.05 M K_2SO_4 (also used for extraction of microbial biomass C) and 2 M KCl (also used for extraction of exchangeable NH_4^+ and NO_3^-). Care needs to be exercised when using salt solutions since concentrations of dissolved organic C measured may well differ from those measured by water extraction which, in turn, could differ from that present in displaced soil solutions. This is principally because dissolved organic matter is in equilibrium with that adsorbed to clay colloids and pH of extraction, ionic strength and dominant species of anions and cations present all affect adsorption/desorption of organic matter (Haynes 2005).

Dissolved organic matter originates from leaching from above- and below-ground plant litter and/or the synthetic activity of soil microflora involved in decomposition of the litter and/or native soil organic matter. Organic matter in soil solution is in equilibrium with solid phase organic matter including (i) that adsorbed to sesquioxide surfaces, (ii) that precipitated due to coagulation in the presence of divalent and trivalent cations and (iii) the insoluble organic matter present in the topsoil (Reemtsma et al. 1999). It consists of a wide range of organic compounds including simple aliphatic organic acids, phenols, phenolic acids, free amino acids, sugar acids, carbohydrates and complex humic molecules of various molecular weights (Stevenson 1994). Only 10–40% (typically about 20%) of dissolved organic matter is readily degradable by soil microflora (Jandl and Sollins 1997; Huang et al. 1998; Smolander et al. 2001) and this fraction is thought to be principally present as carbohydrates (Haynes 2005). The bulk of dissolved organic matter is present as soil humic material that is relatively recalcitrant (Boyer and Groffman 1996; Yano et al. 2000; Wagai and Sollins 2002). Nonetheless, the flux of metabolizable C passing through the labile degradable fraction is a major determinant of soil microbial activity.

Dissolved organic C typically accounts for only 0.05–0.40% of soil organic C in agricultural soils (Campbell et al. 1999a,b; Lundquist et al. 1999) and 0.25–2.0% in forest soils (Boyer and Groffman 1996; Smolander et al. 2001).

Due to the transient nature of dissolved organic matter, seasonal fluctuations in its concentrations are commonly encountered (Sarathchandra et al. 1988; Jensen et al. 1997; Campbell et al. 1999a,b; Murphy et al. 2000). These have been ascribed to seasonal variability in organic inputs through rhizodeposition, microbial death and residue inputs and/or seasonal variability in soil microbial activity and thus metabolism of the

dissolved organic matter. Sampling time is therefore an important consideration when interpreting data.

3.1.3 Extractable Fractions

Many different chemical extractants have been used in attempts to extract a labile portion of organic matter from soils. For example, a large number of chemical reagents have been used to estimate potentially mineralizable N (Keeney 1982; Goh and Haynes 1986). These can be divided into three broad groups: (i) weak (hot water, hot 0.01 $CaCl_2$ or 2 M KCl, 0.01 M $NaHCO_3$), (2) intermediate (alkaline permanganate, Na_2CrO_4 plus H_3PO_4, 1 M NaOH) or (3) strong (6N H_2SO_4, K_2Cr_2 O_7–H_2SO_4) extractants. Numerous other reagents have also been used including NaOH, Na_2CO_3, $Na_2P_2O_7$, acetylacetone and chelating agents. In recent times three different extractants have been frequently used to evaluate labile organic matter. These are the hot water extractable dilute acid-hydrolysable and permanganate-oxidizable fractions.

Hot water-extractable C accounts for 1–5% of soil organic C (Leinweber et al. 1995; Sparling et al. 1998; Chan and Heenan 1999) and about 50% of this is thought to be present as carbohydrate (Haynes 2005). Because it is usually extracted from air-dried soils much of the pool originates from desiccated microbial cells but it also includes exocellular polysaccharides, root exudates, lysates and humic material (Redl et al. 1990; Leinweber et al. 1995; Sparling et al. 1998). Both hot water extractable C (Sparling et al. 1998; Chan and Heenan 1999) and hot water-extractable carbohydrate (Ball et al. 1996; Haynes and Beare 1997; Debrosz et al. 2002) have been used as indices of soil quality.

Dilute acid (0.5 M– 2.5 M H_2SO_4) – extractable C or carbohydrate – C have also been used as indicators of soil organic matter status (Angers and Mehuys 1989; Chan and Heenan 1999; Shepherd et al. 2001). The acid hydrolysable fraction generally accounts for about 20–40% of total organic C (Rovira and Vallejo 2002) and 65–85% of the total soil carbohydrate pool (Puget et al. 1999).

A fraction of organic C oxidizable with 333 mM $KMnO_4$ is another measure of labile organic matter (Blair et al. 1995). This fraction encompasses all those organic components that can be readily oxidized by $KMnO_4$ including labile humic material and polysaccharides (Conteh et al. 1999). It commonly accounts for 15–20% of total soil organic C (Blair et al. 1998; Conteh et al. 1998).

3.1.4 Potentially Mineralizable C and N

Potentially mineralizable C and N are often measured by incubating a sample of field-moist soil at a known temperature in a sealed chamber containing an alkali trap. The CO_2–C accumulated in the trap is measured by acid titration and this represents the quantity of C mineralized. Alternatively, CO_2 in the headspace of the incubation chamber can be measured using a CO_2 analyser. The amount of N mineralized during incubation is calculated as the difference in extractable NH_4^+ - and NO_3^-–N measured in the soil before and after incubation. Mineralizable N can also be measured in an open incubation system where the soil is leached periodically and NH_4^+ - and NO_3^-–N in leachates is measured (Stanford 1982).

Potentially mineralizable C and N are not analogous measurements since the CO_2 evolved during incubation indicates the total metabolic activity of the heterotrophic microflora which are decomposing soil organic matter (i.e. gross C mineralization). By contrast, N mineralization and immobilization occur simultaneously so that a portion of N mineralized during the incubation can be subsequently assimilated (immobilized) by the decomposer microflora. The excess NH_4^+ and NO_3^- accumulates in the soil. Thus, the mineral N accumulated during incubation represents net rather than gross N mineralization.

It has been suggested that estimation of potentially mineralizable C and N is a particularly relevant measurement because it represents a bioassay of labile organic matter using the indigenous soil microbial community to release labile fractions of C and N from soil organic matter under controlled conditions (Sparling 1997). It can, however, be difficult to compare mineralizable C and N values between studies because of differences in soil water content, incubation temperature and period of incubation. Thus, potentially mineralizable C and N values are usually treated as relative rather than absolute values.

As with particulate and soluble organic matter, seasonal fluctuations in potentially mineralizable C and N can occur in field soils (Boone 1994; Bonde and Rosswall 1987; Campbell et al.1999 a,b). These are normally related to seasonal inputs of readily mineralizable organic matter through rhizodeposition of root material during crop growth and/or inputs of litter and crop residues.

3.2 Effects of Agricultural Activity

As noted earlier, total soil organic matter content can be considered as a coarse indicator of soil quality. However, changes in the content of organic C and total N occur only slowly and do not provide an adequate indicator

of important changes in soil organic matter quality that may be occurring. In order to evaluate such changes, the measurement of labile pools of organic matter that make up a relatively small proportion of total soil organic matter (as described above) is required. These pools can be considered as fine indicators of soil quality which influence soil quality in various ways.

Particulate organic matter is widely considered as a key indicator of soil quality (Gregorich and Janzen 1996). Conversion of undisturbed native forest or grassland sites to arable agriculture typically results in a disproportionate decrease in particulate organic matter (Dalal and Chan 2001). This occurs because litter inputs are greatly decreased while their rate of decomposition is increased by factors such as tillage, irrigation and fertilizer inputs. Indeed, agricultural practices that affect the amount of organic residue input and/or the rate of decomposition have much greater and earlier effects on particulate organic matter than whole-soil organic matter content (Biederbeck et al. 1994; Bremer et al. 1994). The greater responsiveness of particulate than total soil organic matter to changes in management have included increases due to (i) continuous cropping compared to summer fallow, (ii) cropping with grasses, legumes and pastures rather than arable row crops, (iii) conversion from conventional to zero tillage and (iv) application of fertilizers thus increasing crop growth and residue input (Janzen et al. 1992; Gregorich et al. 1997b; Angers et al. 1999; Bolinder et al. 1999). The concentrations of particulate organic matter was reduced substantially by cultivation, with the microaggregates showing an almost complete loss of its particulate organic matter content. The destruction of these transient organic cementing agents was assumed to have contributed to the collapse of the macroaggregates. This has resulted in exposure of particulate organic matter, making it more available to rapid oxidation and microbial attack. Particulate organic matter content could be used as indicator of soil structure degradation due to exhaustive cultivation practices (Bongiovanni and Lobartini 2006). Carbon concentrations of light fraction were significantly higher under no-till and forest than under conventional tillage. Soil organic matter loss following conversion from forest to agriculture is attributed to reduction in C concentration in both light and heavy fractions. In contrast, soil organic matter gain upon conversion from conventional tillage to no-till is primarily assigned to an increase in C concentration in the light fraction (Tan et al. 2007). However, some studies disagree with the above results, Leifeld and Kögel-Knabner (2005) found that the proportion of free light (wet sieving, density < 1.8 g cm^{-3}) and occluded light (ultrasonic dispersion with 22 J ml^{-1}, < 1.8 g cm^{-3}) particulate organic matter (POM) showed no clear response to land-use in an agricultural system with sandy dystric Cambisols in Bavaria, Germany.

They concluded that neither free nor occluded light POM are appropriate early indicators for changes in land-use. Some have questioned the validity of dissolved organic matter as an indicator of soil quality because of its small size and highly labile nature (Baldock and Nelson 2000). That is, as explained previously, it is the flux of readily-available substrate through the dissolved organic matter pool that is important in relation to the size and activity of the microbial biomass and nutrient availability. The size of the pool measured at any one time does not necessarily reflect the flux through it.

Nonetheless, the size of the pool of dissolved organic matter has been used successfully as an indicator of changes in soil management. It has been shown to increase more markedly than total organic matter content due to (i) addition of crop residues, (ii) replacement of wheat-fallow systems with continuous wheat, (iii) conversion of arable systems to pasture and (iv) stock camping of grazing animals (Campbell et al. 1999a,b; Haynes 1999, 2000; Haynes and Williams 1999; Graham et al. 2002). For example, DOM concentration was reported to decrease in the order: forest floor > grassland A_h> arable A_h. Although land use and management practices may significantly influence the amount and the composition of DOM in soil, the processes involved remain largely unknown. Quite a lot of previous results were obtained from laboratory studies which may help to isolate and better define these processes, but the net effect of management practices can be poorly predictable under field conditions because various soil properties are modified at the same time, resulting in confounding and counteracting effects on DOM (Chantigny 2003).

The 0.05 M K_2SO_4 – tractable (soluble) organic C content of the seven land uses referred to in Sect. 3 is shown in Fig. 1. It is evident that annually-tilled ryegrass and maize and sugarcane have the lowest values suggesting they had the lowest concentrations of soluble substrate C for microbial growth and activity at the time of sampling.

Extractable fractions of organic matter have also been employed to guage the effects of soil management on soil organic matter quality. Hot water-extractable carbohydrate has been shown to be more responsive to inclusion of short-term pasture in arable rotations and to rhizosphere versus bulk soil than total carbohydrate or organic C content (Haynes et al. 1991; Haynes and Francis 1993). Similarly, dilute acid-hydrolysable carbohydrate has been shown to be more responsive to changes in management than organic C content (Angers and Mehuys 1989; Angers et al. 1993 a,b). The $KMnO_4$ – oxidizable fraction has been shown to be more sensitive than organic C to (i) conversion from grassland to arable agriculture and (ii) conversion from burning to crop residue retention (Blair et al. 1995, 1998; Conteh et al. 1998; Blair 2000).

The pools of potentially mineralizable C and N show a greater responsiveness to changes in soil management than do organic C or total N (Campbell et al. 1997; Gregorich et al. 1997a). Disproportionately greater increases in mineralizable than total organic matter have been noted in responses to (i) decreases in the amount of fallow in cereal rotations, (ii) cropping with grasses rather than cereals, (iii) conversion from conventional to zero tillage, conversion from burning to retention of crop residues and (iv) long-term fertilizer applications (Carter and Rennie 1982; Biederbeck et al. 1994; Bremer et al. 1994; Campbell et al., 1997; Needelman et al. 1999; Graham et al. 2002). Potentially mineralizable N concentrations are often less responsive to soil management than those of C because temporary immobilization of N can occur concomitantly with C mineralization and release of CO_2 (Campbell et al. 1997).

4 Size and Activity of the Soil Microbial Biomass

4.1 Nature of the Pools

4.1.1 Microbial Biomass

The soil microbial biomass is the living component of soil organic matter. It comprises mainly bacteria and fungi and excludes soil animals and plant roots. Although the soil microbial biomass only consists of 1–5% of organic C and 1–6% of total N (Sparling 1997; Dalal 1998) it performs critical functions in the soil system. It is a labile source of C, N, S and P following microbial death and subsequent mineralization, an immediate sink of C, N, S and P through immobilization and it is an agent for nutrient transformations and pesticide degradation. Indeed, the diverse metabolic activities of the soil microbial community regulate energy and nutrient cycling that takes place in the soil. In addition, the microbial biomass can play an important role in regulating soil structure. Microbial mucigel contains polysaccharides and these act as glues thus helping bind aggregates together (Tisdall 1996). In addition, fungal hyphae can have an enmeshing effect helping bind soil aggregates together (Tisdall 1996).

Historically, measurement of the microbial biomass has been a tedious, time-consuming occupation involving staining and direct counting or use of culture media and enumeration of individual microbial communities. However, in the last 20 years, a suite of methods have been developed for more rapid assessment of the microbial biomass. These include the substrate-induced respiration method (Anderson and Domsch 1978), the chloroform fumigation-incubation method (Jenkinson and

Powlson 1976), the chloroform fumigation-extraction method (Vance et al. 1987), adenosine triphosphate analysis (Jenkinson et al. 1979) and phospholipid fatty acid analysis (Zelles 1999). The most common method used routinely is the fumigation-extraction method. It involves measurement of 0.05 M K_2SO_4-extractable organic C from unfumigated and chloroform-fumigated soil. Chloroform fumigation causes death and lysis of soil microbes. The proportions of C (K_C) and N (K_N) extracted from the fumigated soil varies from 0.20 to 0.68. Most frequently used K_C values range from 0.36–0.45 and K_N values are in the range of 0.49–0.62.

Seasonal fluctuations in soil microbial biomass can occur due to changes in the amount of substrates, and temperature and moisture (Dalal 1998). Indeed many workers have recorded seasonal fluctuations in micro-microbial biomass under both pastoral and arable conditions (Sarathchandra et al. 1989; Tate et al. 1991; Srivastava 1992; He et al. 1997; Campbell et al. 1999). Such seasonal variations must be taken into account when microbial biomass data is compared.

4.1.2 Basal Respiration

Measuring the size of the microbial biomass gives no indication of its activity. This is because the soil microbes can be present in resting, inactive forms or in metabolically active forms. The activity of the microbial community is most commonly estimated by measuring the soil respiration rate (usually as CO_2 evolution rate). Under field conditions, soil respiration rates are characteristically variable and can show wide variation depending on such factors as soil water content, temperature and substrate availability (Sparling 1997). It is therefore often difficult to interpret field measurements. For that reason, measurement of the respiration rate is commonly made under controlled laboratory conditions when soil water content and temperature are not limiting. Such values are normally termed “basal respiration” and give an indication of microbial activity.

Measurements other than respiration rate can also be used as indicators of soil microbial activity. These include measurements of the rate of multienzyme processes such as arginine ammonification rate (Alef and Kleiner 1995) fluorescein diacetate (FDA) hydrolysis rate (Alef 1995) and measurement of key endocellular enzymes such as dehydrogenase (Tabatabai 1994).

4.1.3 Metabolic Quotient

From the measurements of microbial biomass C and basal respiration the metabolic quotient (qCO_2) can be calculated. This is a measure of microbial

respiration per unit of microbial biomass (i.e. μg CO_2–C h^{-1} mg^{-1} microbial biomass). Calculating the qCO_2 gives an indication of what proportion of substrate C is respired as CO_2 rather than incorporated into cellular C. A high qCO_2 is thought to indicate that the microbial community is stressed and is tending to respire C as CO_2 rather than incorporating it into their biomass (Anderson and Domsch 1993; Wardle and Ghani 1995).

It has been suggested (Insam and Haselwandter 1989; Anderson and Domsch 1990) that the metabolic quotient can be used as an index of ecosystem development. As an ecosystem reaches maturity, selection pressure towards efficient use of available resources results in a larger microbial biomass with a lower metabolic quotient. Nonetheless, Wardle and Ghani (1995) concluded that qCO_2 can be insensitive to disturbance and ecosystem development, fails to distinguish between effects of disturbance and stress and does not decline predictably in response to ecosystem development wherever stress increases along successional gradients.

4.2 Effects of Agricultural Activity

The rate of turnover of the microbial biomass is typically 0.2–6 years compared to greater than 20 years for the bulk of organic matter (Jenkinson 1990). Due to its dynamic nature, the microbial biomass serves as a sensitive indicator and early predictor of changes in soil organic matter status (Powlson and Jenkinson 1981; Powlson et al. 1987) induced by changes in management practices such as crop residue management, tillage practice (Carter 1986) and the use of grass leys in rotation (Haynes et al. 1991).

Microbial biomass C has been shown to increase more markedly than organic C content in response to (i) addition of crop residues (Ocio et al. 1991; Venkateswarlu et al. 2007), (ii) conversion of arable systems to pasture (Haynes et al. 1991; Haynes and Francis 1993), (iii) an increasing proportion of forages in arable rotations (Dalal 1998; Angers et al. 1999) (iv) conversion from conventional to zero tillage (Bolinder et al. 1999; Dominy and Haynes 2002) and (v) stock camping by grazing animals (Haynes and Williams 1999) and decrease more markedly than organic C in response to conversion to arable cropping (Haynes and Tregurtha 1999; Dominy and Haynes 2002). For example, Böhme et al. (2005) observed increases of microbial biomass C in the farmyard manure-fertilized soils, while NPK fertilization significantly decreased soil microbial biomass C.

Ros et al. (2006) reported that compost treatments (urban organic wastes, green wastes, manure and sewage sludge) resulted in an increase of microbial biomass carbon and basal respiration in Austrian soils with long-term crop rotation. Application of farmyard manure also increased microbial biomass carbon in a calcareous soil in northwest China (Yang et al. 2007). Cumulative pig slurry addition had a significant stimulating effect on microbial biomass carbon content in soils of Santa Olalla, Spain. No significant effect of pig slurry amendment on total organic C, water soluble organic C and basal respiration was detected (Hernández et al. 2007). A 13 years of inorganic fertilizer application in paddy soil showed that microbial biomass was significantly higher in the treatments fertilized with P than those in the treatments without P fertilization. The significant effects of P fertilizer were mainly ascribed to the enhanced growth of rice crops and accumulation of soil organic carbon through increased root turnover and rhizodeposition (Zhong and Cai 2007). Cultivation of soils in the central highlands of Mexico with maize reduced microbial biomass C. Converting soil under natural vegetation to arable soil was not only detrimental for soil quality, but might be unsustainable as organic matter input is limited (Reyes-Reyes et al. 2007).

Results in Fig. 2 show a comparison of organic C and microbial biomass C down the profile to a depth of 30 cm in sugarcane fields that are either under preharvest burning or green cane harvesting with retention of a mulch of crop residues at the soil surface. Samples were taken either in the row (below the plant stools) or in the middle of the fallow inter-row area. Results show there is a significant accumulation of organic C in the row, compared to the inter-row to a depth of 20 cm under both managements. In addition, there was a significant accumulation of organic C under residue return to a depth of 10 cm in both areas of the field. These effects were much magnified when microbial biomass C was measured. Significant increases were noted to a depth of 30 cm due to both residue return and sampling in the row rather than the inter-row.

Because of the greater responsiveness of microbial biomass than organic C to changes in soil management, the percentage of organic C present as microbial biomass (sometimes termed the microbial quotient) can be used as a useful indicator (Sparling 1992, 1997). In general, if a soil is being used exploitively and it is losing organic matter, the microbial quotient is lowered. By contrast, where soils are rapidly accumulating organic matter the microbial quotient will be increased. It is evident from Fig. 1 that of the 7 land uses discussed previously, the microbial quotient was lowest under annually tilled ryegrass and maize and sugarcane. Trends were, as expected similar to those for K_2SO_4 – extractable C.

Calculation of the microbial quotient can make treatment effects much more obvious. For example, in Fig. 2 the microbial quotient was greater in the row than inter-row and greater under residue return than burning, both to a depth of 30 cm. In addition, values declined with depth as soil organic matter content declined and the proportion of organic C present in labile form declined.

Since the basal respiration is measured as CO_2–C evolved during a laboratory incubation, it is essentially the same measurement as potentially mineralizable C (see above). As noted previously, this measurement shows greater responsiveness than total organic C to changes in soil land use/management. This is to be expected since labile organic matter fractions are more responsive than organic C content to changes in management and microbial activity is greatly influenced by the amount of labile, readily metabolizable organic matter present.

The qCO_2 is often used as an indicator of whether the microbial biomass is under stress. In general, factors that decrease the size of the microbial biomass tend to increase qCO_2. That is, factors that cause stress to the microbial community tend to reduce its size. Other factors could also contribute to an increased qCO_2. For example, bacterial communities are less efficient at converting substrate C into cellular C than fungi (Sakamoto and Oba 1994) so a change in the composition of microbial biomass can alter qCO_2 values.

Certainly, calculation of the metabolic quotient can reveal trends very different from those of basal respiration. As shown in Fig. 1, for the 7 land uses, trends in basal respiration were broadly similar to those for microbial biomass C and organic C. However, when the metabolic quotient was calculated, trends with land use were very different. Values were greater under sugarcane, maize and to a lesser extent annual ryegrass, than the other treatments. This suggests that the microbial community under these arable land uses is under more stress and/or has a different composition to that under the others. The most likely microbial stress under these land uses is likely to be a shortage of available substrate C.

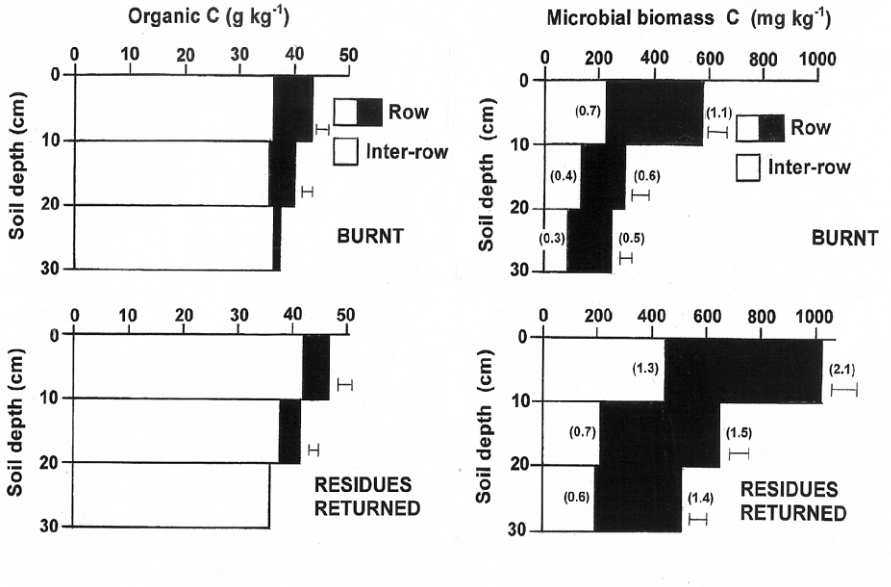

Fig. 2. Distribution of organic C and microbial biomass C in the soil profile under sugarcane annually preharvest burnt or green cane harvested with retention of crop residues on the soil surface. Soils were sampled from below the plant row or in the centre of the inter-row area. Values for microbial quotient are shown in parenthesis. LSD ($P \leq 0.05$) shown. (From Haynes, unpublished, 2005).

Wardle and Ghani (1995) reviewed data on the effects of management practices on qCO_2. They concluded that some practices, such as liming and fertilizer application can either increase or decrease qCO_2 values depending on whether the disturbance alleviates the stress (reducing qCO_2) or is more extreme than the stress it alleviates (enhancing qCO_2). Although tillage represents a severe disturbance, Wardle and Ghani (1995) found that qCO_2 was not predictably enhanced by this perturbation. In addition,

when there is an increase in qCO_2, the reason is not necessarily clear. For example, Nsabimana et al. (2004) found that in comparison with other long-term land uses, arable cropping resulted in an increase in qCO_2.

However, it also resulted in an increase in FDA hydrolytic activity and arginine ammonification rate when expressed per unit of microbial biomass. They suggested that arable management resulted in a small microbial community that has a high metabolic activity. By contrast, other land uses had a larger microbial biomass but with a larger proportion of the community present in resting and other non-metabolically active forms. Overall, it does not seem that qCO_2 can be used as a universal indicator of a stressed microbial community.

5 Conclusions

The nature and significance of the various parameters of soil organic matter quality and microbial activity discussed in this paper are summarized in Table 1. The multifunctional role of soil organic matter, as illustrated in Table 1, means that a suite of labile fractions is typically required to provide an overview of major soil functions including structural condition, nutrient availability and soil biological activity. In general, agricultural practices that affect the amount of input of organic matter and/or its rate of decomposition, greatly have a much greater and earlier effect on labile fractions of organic matter that whole-soil organic matter content. Thus, changes in these fractions provide an indication of changes in total soil organic matter content that may become evident in the long-term. Concentrations of labile soil organic matter in soils can be subject to seasonal variability so that sampling needs to be carried out at the same time every year. Similarly, the size and activity of the microbial biomass can change rapidly in response to changes in C status but can also undergo seasonal variability.

Because different fractions of organic matter reflect the key functions of soil organic matter, their measurement is useful in investigating how various agricultural management practices influence the biological, chemical and physical properties of soils and ultimately the sustainability of such practices. Similarly, the size and activity of the microbial community impinges on many soil properties and processes. With an understanding of how soil management effects soil properties and processes, new strategies can be devised that will improve agricultural sustainability.

Table 1. Nature, significance and typical quantities of various measures of organic matter quality and the size and activity of the microbial biomass present in soils

	Typical quantities	Nature and Significance
Total organic C and N	Organic C = 7–60 gC kg^{-1} Total N = 0.6–5.0 gN kg^{-1}	Sum of organic material (both living and dead) present in soil excluding living plant material. Single most important factor involved in soil productivity. Has massive effects on chemical, physical and biological properties and processes in soils.
Particulate organic matter	LF = 2–18% of organic C, 1–16% of total N SSF = 20–45% of organic C and 13–40% of total N	Partially decomposed plant litter isolated by density fractionation (LF) or sieving (SSF). Substrate and centre for soil microbial activity, short-term reservoir of nutrients, food source for earthworms and other soil fauna and focci for formation of water stable aggregates.
Soluble organic matter	About 0.05–0.40% organic C and total N	Water soluble organic compounds present in soil solution including simple compounds of plant and microbial origin.
Extractable organic C and N	Variable amount of organic C (1– 40%) depending on the extractant	Organic C and N solubilized/hydrolyzed/oxidized by various chemical reagents. The hot water-extractable fraction is dominated by microbial carbohydrates and is believed to be involved in aggregate stabilization. Acid-hydrolyzable carbohydrates are also thought to be involved in aggregation. Permanganate-oxidizable C is a non-specific labile fraction.

Table 1. (Continued)

	Typical quantities	Nature and Significance
Potentially mineralizable C and N	About 1–5% of organic C and total N	Quantities of organic C and N released by indigenous soil microflora during a laboratory incubation. Values are the result of an integration of physical, chemical and microbiological properties of the soil. Indicator of the N fertility of soils and their ability to supply N to crops.
Microbial biomass	1–5% of organic C and 1–6% of total N	Organic material associated with cells of living soil microorganisms. Agent for transformation and cycling of organic matter and nutrients, formation and decay of humic material, dynamic source and sink of plant nutrients and an agent involved in formation and stabilization of aggregates.
Basal respiration	Variable depending on incubation Conditions. Often in the range of 5–40 μg CO_2–C g^{-1} day^{-1}	Quantity of organic C released as CO_2 during an aerobic laboratory incubation. A measurement of the activity (respiration rate) of the soil microbial community.
Metabolic quotient	Variable; often in the range of 2–40μg CO_2–C mg^{-1} biomass day^{-1}	Basal respiration per unit of microbial biomass C. Provides a useful measure of the efficiency with which microbes are using substrate C. Often used as an index of the degree of microbial stress.

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9 Soil Carbon Dynamics in a Subtropical Mountainous Region, South China: Results Based on Carbon Isotopic Tracing

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1 Introduction

Carbon cycling within the terrestrial ecosystems is predominant as the most uncertain component in the global carbon cycles (Houghton et al. 1998; Steffen et al. 1998), and is therefore critical in global carbon budgeting (Trumbore et al. 1996; Rosenzweig and Hillel 2000). A large portion of terrestrial carbon resides in soil organic carbon (Malhi et al. 1999; Garten et al. 2000), and carbon storage in soils can be increased by reforestation of agricultural land (Binkley and Resh 1999; Scott et al. 1999) and by the effective management of existing forests (Johnson and Curtis 2001). It is then pressing to decipher soil carbon dynamics for the soils in different climate regimes, due to the Kyoto Protocol (UNFCCC 1997).

Soil contributes to a greater extent to total carbon storage than do above-ground vegetation in most forests (Johnson and Curtis 2001). The total amount of soil organic carbon (SOC) in the upper meter of soil is about 1500×10^{15} g C (Eswaran et al. 1993; Batjes 1996), and the global atmospheric pool of CO_2 is about 750×10^{15} g C (Harden et al. 1992). The CO_2 emission from soil into atmosphere is about $68.0–76.5 \times 10^{15}$ g C per year, and this is more than 10 times the CO_2 released from fossil fuel combustion (Raich and Potter 1995). Variations in SOC pools and SOM turnover rates, therefore, exert substantial impacts on the carbon cycles of terrestrial ecosystems in terms of carbon sequestration in soil and CO_2 emission from soil.

The distribution of SOC with depth is attributed mainly to continuous input and decomposition of soil organic matter (SOM), and correlates directly with soil development and SOM turnover (Chen et al. 2005). Regional, continental or global models are useful to understand SOM dynamics according to land use changes and management practices (Cole et al. 1996). These models require a thorough knowledge of the distribution of C in different soils and under different land uses practices (Paustian et al. 1997). Quantification of changes in soil carbon dynamics, including SOM turnover rate and distribution of SOC with depth, is therefore critical for determining carbon storage in soils and for modeling soil carbon cycling.

The use of natural ^{13}C abundance to determine SOM turnover associated with land management (Balesdent et al. 1988; Follett et al. 1997; Collins et al. 1999) and climate changes (Loiseau and Soussana 1999; Hobbie et al. 2002, 2004) is gaining popularity. $\delta^{13}C$ analysis has become a valuable measure in the study of SOM dynamics (Bird et al. 1996), especially in the regions with records of vegetation shifts between C_3 and C_4 species (Gregorich et al. 1995; Ineson et al. 1996; Boutton et al. 1998; Collins et al. 1999, 2000). The changes in isotopic composition of soil

with known and dated vegetation changes are directly related to SOM dynamics (Balesdent 1987, 1990; Martin et al. 1990; Garten et al. 2000).

SOM $\delta^{13}C$ values correlate well with SOM sources, SOM composition and turnover processes during soil development (Balesdent et al. 1993; Chen et al. 2002a; Powers and Schlesinger 2002; Wynn et al. 2005). The changes in $\delta^{13}C$ of SOM with depth have several possible explanations (Balesdent et al. 1990; Wynn et al. 2006). One popular explanation is the effect of carbon isotope fractionation due to preferential decomposition of SOM components with different isotopic composition (Benner et al. 1987; Wedin et al. 1995) and kinetic fractionation of carbon isotopes through microbial respiration of CO_2 during SOM decomposition (Mary et al. 1992; Macko and Estep 1984). The spatial and temporal variations of SOM $\delta^{13}C$ in relation to SOM turnover are then effective proxies for deciphering SOM dynamics.

Soil layers with positive SOM $\Delta^{14}C$ values contain ^{14}C produced by nuclear weapon testing ("bomb ^{14}C") from the 1950s to the 1960s, and the maximal depth that "bomb ^{14}C" reaches is called "bomb ^{14}C" penetrating depth (Shen et al. 2001). The ^{14}C dating results measured with total soil organic carbon are usually prone to be younger, due to addition of new organic carbon during pedogenesis. This kind of ^{14}C dating result is generally called to be SOM ^{14}C apparent age (Shen et al. 2000). The SOM ^{14}C apparent ages of the upper soil layers with SOM $\Delta^{14}C$ greater than 0, which can not be obtained directly from measurement, can now be calculated based on SOM ^{14}C budget model (Chen et al. 2002b).

Little is known about the effect of leaching on distribution of SOM with depth, which is unfavorable for evaluating the potential capacity of soil to sequester carbon. Sporopollen (pollen and spores) are abundant in upper soils, and their vertical distributions are controlled substantially by leaching (Zheng et al. 2002). The distribution of sporopollen with depth may be a useful index of leaching potential. We intended to evaluate the effect of leaching on SOM vertical distribution, based on variations in SOC concentration and SOM ^{14}C apparent age with depth. The distribution of sporopollen with depth can serve as a reference for our evaluation.

Five soil profiles at different elevations with specific vegetation composition were selected at the Dinghushan Biosphere Reserve (DHSBR), South China, and soil samples were taken using the thin-layered method (Becker-Heidmann and Scharpenseel 1986). Our aims were to study the spatial and temporal variations of SOM along an altitudinal gradient at the DHSBR that may serve as a substitution of different climate zones, based on SOC concentrations, SOM ^{14}C dating, SOM $\delta^{13}C$ values and sporopollen

abundance of the soil samples. Studies on SOM dynamics along an altitudinal gradient in a mountainous region may present clues for deciphering soil carbon cycling in different climate regimes.

2 Materials and Methods

2.1 Study Sites

The study sites were located at the Dinghushan Biosphere Reserve, South China (23°09′21″–23°11′30″N, 112°30′39″–112°33′41″E), with southern subtropical monsoonal humid climate. The average annual temperature is 21ºC, annual precipitation is about 1900 mm, and wet and dry seasons are well defined at the DHSBR. The wet season is from April to September, and the dry season is from November to January (Deng et al. 1990; Yu and Peng 1995). The local vegetation is tropical monsoonal rain forest and subtropical monsoonal evergreen broad-leaved forest, and the DHSBR is representative of the southern subtropical forest ecosystem in China (Tu 1984; Deng et al. 1990). The DHSBR was selected as a Forest Ecosystem Station, joining the UNESCO-MAB Biosphere Reserve Network in 1979 (Tu 1984). Consequently, the area has had little human disturbance since 1979.

Jilong Mountain, in the northwest part and with an elevation of 1000 m a.s.l., is the highest peak of the DHSBR. Additional site characteristics of the DHSBR are reported in Shen et al. (1999). The basic site characteristics of the studied profiles are provided in Table 1. Thin-layered sampling (Becker-Heidmann and Scharpenseel 1986) was conducted to collect 1.5–2.0 kg of soil from each sampling section (Table 2). All soils within the sampling intervals were collected to obtain bulk samples. The selection of sampling intervals (Table 2) was based on soil characteristics and the need to recover the penetrating depth of "bomb ^{14}C" (Shen et al. 1999) and to determine the variation of SOM $\delta^{13}C$ with depth. All plant debris within a plot of 0.4 m × 0.4 m was sampled by hands near the soil profiles, and sealed in plastic bags.

Table 1. Site characteristics of the selected soil profiles with different elevations at the Dinghushan Biosphere Reserve, South China

Profile	Profile thickness (m)	Elevation (m a.s.l.)	Slope direction	Slope degree	Vegetation	Sampling date
JLS	0.7	1000	SE173°	30°	Meadow	July 1998
GC	0.6	905	NE75°	16°	Shrub, dominated by *Rhododendron sinsii* Planch. and *Corydalis pallida* Pers., with a little herbage dominated by *Aristida chinensis* Munro and *Eriachne pallescens* R. Br.	July 1998
SL	1.1	662	NE42.5°	30°	Coniferous & broad-leaved mixed forest (natural forest), consists mainly of *Engelhardtia chrysolepis* Hance, *Bridelia monoica* (Lour.) Merr., and *Machilus velutina* Champ.	July 1998
WKS	1.6	315	NE50°	22°	Coniferous & broad-leaved mixed forest (natural forest), consists mainly of *Schima superba* Gardn. et Champ., *Castanopsis chinensis* Hance, *Craibiodendron kwangtungense* S. Y. Hu, and *Castanopsis fissa* (Champ.) R. et W.	May 1996
QYS	1.6	190	NE73°	37°	Monsoon evergreen broad-leaved forest (natural forest), dominated by *Castanopsis chinensis* Hance, *Schima superba* Gardn. et Champ., *Cryptocarya chinensis* (Hance) Hemsl., *Cryptocarya concinna* Hance, and *Lindera chunii* Merr.	May 1996

Table 2. Soil sampling design of the selected soil profiles at the Dinghushan Biosphere Reserve, South China

Profile	Sampling section (m)	Sampling interval (m)	Number of samples
JLS	0–0.2	0.02	10
	0.2–0.45	0.05	5
	0.5–0.7	0.2	1
GC	0–0.3	0.02	15
	0.3–0.4	0.05	2
	0.4–0.6	0.2	1
SL	0–0.4	0.02	20
	0.4–0.6	0.2	1
	0.6–0.9	0.1	3
	0.9–1.1	0.2	1
WKS	0–0.2	0.05	4
	0.2–0.8	0.1	6
	0.8–1.6	0.2	4
QYS	0–0.4	0.05	8
	0.4–0.8	0.1	4
	0.8–1.6	0.2	4

The meadow profile (JLS) was located on the peak of Jilong Mountain, the slope direction of the excavating site was 173°SE, and slope angle 30°. The ground was covered by plants, and a weathering crust was encountered at the bottom of the 0.7-m deep profile. The 0–0.2 m horizon was greyish brown, with abundant plant roots; 0.2–0.3 m was brownish yellow transitional layer, with sand concentration increasing with depth; 0.3–0.7 m was brownish yellow, intermingled with light grey sandy specks in size of 0.02–0.03 m. The detailed descriptions for shrub-meadow profile (GC) and forest profile (SL) are reported in Chen et al. (2002a), and Wukesong profile (WKS) and Qingyunsi profile (QYS) are reported in Shen et al. (1999, 2000). Based on the loose topsoil with abundant plant debris and fine grass roots, it is believed that there has been little recent erosion of topsoil on the steep slopes. The studied soil profiles corresponded to Ferralsols in the

FAO classification (FAO 1998). Soil sampling design and the number of samples collected from each profile are provided in Table 2.

2.2 SOM ^{13}C Analyses

Methods for pretreatment of samples for ^{13}C analysis had been described in Chen et al. (2002a). After pretreatment, soil samples and plant debris were sent to the State Key Laboratory of Loess and Quaternary Geology, Chinese Academy of Sciences (CAS), for ^{13}C analyses. The ^{13}C analyses were conducted using a Finnigan MAT-251 mass spectrometer manufactured by Finnigan-Mat Company, with a precision of 0.2‰. Results are reported as $\delta^{13}C$, in parts per thousand of the $^{13}C/^{12}C$ ratio from that of the International Pee Dee belemnite (PDB) standard, where:

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] * 1000.$$

2.3 SOM ^{14}C Analyses

Procedures for pretreatment of soil samples and synthesis of sample benzene for ^{14}C analysis had been described in Chen et al. (2002b). Sample benzene was often left for 3–4 weeks to allow any radon with half-life of 3.82 days that may be present to decay. ^{14}C activity of the C_6H_6 was then determined using a 1220-QUANTULUS ultralow-level liquid scintillation spectrometer manufactured by WALLAC Company, Sweden. The ^{14}C analyses were conducted at the Guangzhou Institute of Geochemistry, CAS. Results are reported as $\Delta^{14}C$, in parts per thousand of the $^{14}C/^{12}C$ ratio from that of the standard (oxalic acid decay corrected to 1950) (Stuiver and Polach 1977), and corrected for "bomb ^{14}C" (Chen et al. 2002b), where:

$$\Delta^{14}C = [(^{14}C/^{12}C)_{sample}/(^{14}C/^{12}C)_{standard} - 1] * 1000.$$

SOM turnover rate (m) (year^{-1}) was then calculated based on the methods in Chen et al. (2002b), and SOM apparent age (T) was obtained as $1/m$ (year). Due to the less magnitude of m values, variations of T values with depth are often evaluated to show the variations of SOM turnover rates with depth in subsequent analyses.

2.4 Soil Organic Carbon Concentration

Soil organic carbon concentration was determined by the sulfuric acid-potassium dichromate method (Kalembasa and Jenkinson 1973) at the Soil Chemistry Laboratory of South China Institute of Botany, CAS. Results

are reported as weight percentages, and the measurement error is less than 0.04%.

2.5 Sporopollen Analysis

Pollen and spore samples were extracted by treatment with acid and alkali and floatation with heavy liquid (Faegri and Iverson 1989; Moore et al. 1991). After the pretreatment with hydrochloric acid (HCl) and sodium hydroxide (NaOH), pollen and spore grains were extracted by centrifugal separation of the resulting residue with two to three changes of heavy liquid (potassium iodide). The grain samples were placed on specific glass slides, and then identified and counted with a light microscope at 400× magnification. More than two pieces of slides with pollen and spores were identified for each soil sample. All the grains on the slides were identified and counted, due to the low abundance of sporopollen in the soils. The sporopollen abundance in grains g^{-1} was calculated based on volume methods. Sixteen soil samples of SL profile were selected for this analysis.

3 Results

3.1 SOM $\delta^{13}C$ Values

SOM $\delta^{13}C$ vs depth curves have similar patterns of variation for the soil profiles with different elevations at the DHSBR (Fig. 1). SOM $\delta^{13}C$ value is typically lowest at the surface and becoming richer in ^{13}C with depth, often reaching a maximal value at 0.1–0.3 m depth. In general, below this depth, SOM became gradually depleted in ^{13}C, and SOM $\delta^{13}C$ tended towards a stable value (Fig. 1). The depth with the maximal SOM $\delta^{13}C$ value of JLS, GC, SL, WKS and QYS profiles is 0.15 m, 0.12 m, 0.24 m, 0.18 m and 0.38 m, respectively, showing an increasing tendency with decreasing of elevation (Fig. 2). SOM $\delta^{13}C$ values become enriched in ^{13}C with depth rapidly from the surface of the soil profiles, the greatest enriching rates occur above certain depth, which is 0.08 m, 0.14 m, 0.16 m and 0.22 m for GC, SL, WKS and QYS profiles, respectively (Fig. 1). SOM $\delta^{13}C$ values of JLS profile do not show rapid enrichment in ^{13}C with depth from the surface.

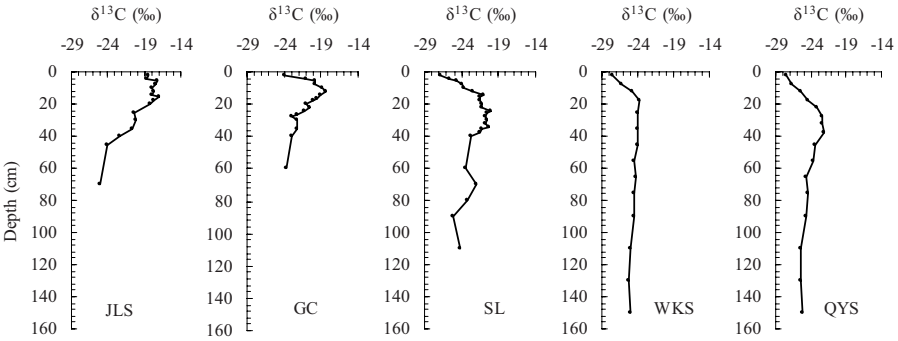

Fig. 1. SOM $\delta^{13}C$ versus depth curves for the soil profiles with different elevations at the Dinghushan Biosphere Reserve.

SOM $\delta^{13}C$ of topsoil becomes enriched in ^{13}C with elevation at the DHSBR (Fig. 3). SOM $\delta^{13}C$ of the topsoil of JLS profile indicates the dominant influence of C4 type vegetation (Fig. 1), and is obviously less than $\delta^{13}C$ of plant debris (Fig. 3). Except for JLS profile, SOM $\delta^{13}C$ of topsoil is greater than $\delta^{13}C$ of plant debris (Fig. 3). The increments in SOM $\delta^{13}C$ of topsoil relative to $\delta^{13}C$ of plant debris do not show regular variations with elevation at the DHSBR (Fig. 3). There exists clear discrepancy between SOM $\delta^{13}C$ of topsoil and the maximal SOM $\delta^{13}C$ of one soil profile (Table 3). Except for JLS profile, this discrepancy is 3.5‰–7‰ for the four profiles with conifer and broad-leaved vegetation, and is not consistent between these soil profiles at the DHSBR (Tables 1, 3).

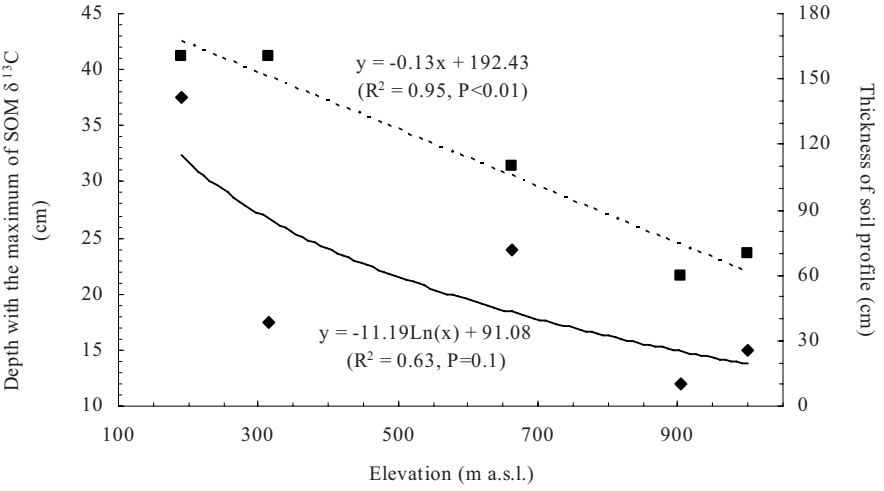

Fig. 2. Depth with the maximum of SOM $\delta^{13}C$ value (diamond) and thicknesses (square) of the soil profiles with different elevations at the Dinghushan Biosphere Reserve.

3.2 SOC Concentrations

SOC concentration decreases exponentially with depth from the maximal value of the topsoil for the soil profiles at the DHSBR (Fig. 4). The SOC concentrations of soil profiles with shrub or herbaceous vegetation, i.e. GC profile and JLS profile, are obviously less than those of other three profiles with conifer and broad-leaved vegetation (Fig. 4). SOC concentrations decrease with depth rapidly from surface of the soil profiles, the greatest decreasing rates occur above certain depth that is correspond to the depth indicating the rapid enrichment of SOM $\delta^{13}C$ in ^{13}C for GC, SL, WKS and QYS profiles (Figs. 1, 4), respectively.

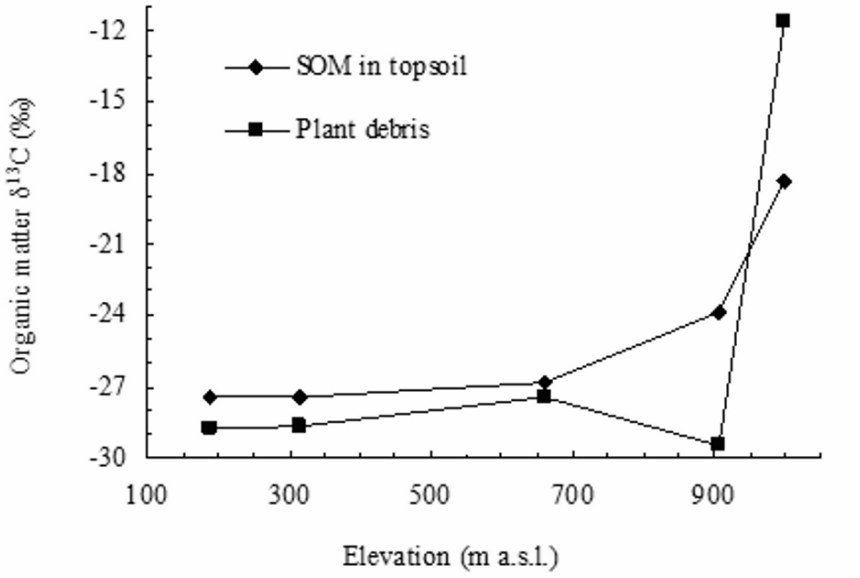

Fig. 3. $\delta^{13}C$ values of plant debris and SOM in topsoil of the soil profiles with different elevations at the Dinghushan Biosphere Reserve.

3.3 SOM ^{14}C Dating

SOM ^{14}C apparent ages (Xing et al. 1998; Shen et al. 2000, 2001; Chen et al. 2002b) show an increasing tendency with depth for the soil profiles along an elevation gradient at the DHSBR (Fig. 5), suggesting that SOM compartments with greater turnover rates were predominant in the upper soils and compartments with less turnover rates were main SOM components in the deep of soil profiles. The calculated SOM ^{14}C apparent ages for the upper soil layers with positive SOM $\Delta^{14}C$ values show also increasing trend with depth (Fig. 5). These calculated values were generally less than 300 years, suggesting that the upper young soil sections were susceptible to contamination of "bomb ^{14}C".

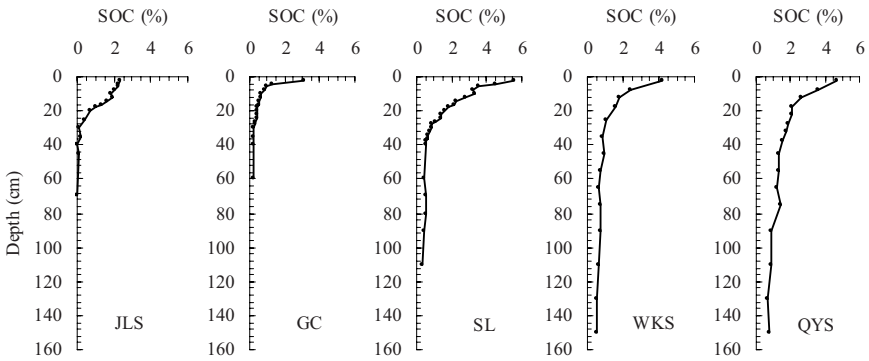

Fig. 4. SOC concentration versus depth curves for the soil profiles with different elevations at the Dinghushan Biosphere Reserve.

The "bomb ^{14}C" penetrating depth of JLS, GC, SL, WKS and QYS profiles is 0.25 m, 0.14 m, 0.3 m, 0.2 m and 0.1 m, respectively. This depth is not consistent with the depth with the maximal SOM $\delta^{13}C$ value for the studied soil profiles at the DHSBR.

3.4 Distributions of Pollen and Spores with Depth

The pollen abundance shows decreasing trend with depth for SL profile, so does the total abundance of pollen and spores (Table 4). The greatest abundance of pollen and spores, 7300 grains g^{-1}, occurs in the upper section (0–0.14 m). The total abundance of pollen and spores decreases with depth from 0.14 m to 0.4 m (Table 4), and the grain number counted during identification was still more than 100 grains for each sample. The abundance of pollen and spores is less than 80 grains g^{-1} below 0.4 m, and only several grains g^{-1} in the lower part of SL profile.

The abundance of pollen decreases rapidly with depth in the upper 0.14 m of SL profile (Table 4). Pollen was scarce below 0.14 m, with abundance of only 0–6 grains g^{-1}, and no pollen was found below 0.26 m. Only modern fern spores were found below 0.26 m, including mainly *Dicranopteris*, *Hicriopteris*, *Microlepia* and other monolete and trilete spores, and turned to be monotonous with depth (Fig. 6).

Table 3. SOM $\delta^{13}C$ of topsoil ($\delta^{13}C_{topsoil}$), the maximum SOM $\delta^{13}C$ value ($\delta^{13}C_{maximum}$) and SOM ^{14}C apparent ages of the bottom of soil profiles with different elevations at the Dinghushan Biosphere Reserve, South China

Profile	Vegetation type	$\delta^{13}C_{topsoil}$ (‰)	$\delta^{13}C_{maximum}$ (‰)	$\delta^{13}C_{maximum}$– $\delta^{13}C_{topsoil}$ (‰)	SOM ^{14}C apparent ages of the bottom (yr B.P.)
JLS	Meadow	–18.32	–16.87	1.45	12786
GC	Shrub	–23.88	–18.33	5.55	2400
SL	Natural forest	–26.88	–20.0	6.88	6690
WKS	Natural forest	–27.40	–23.87	3.53	5572
QYS	Natural forest	–27.46	–22.20	5.26	8750

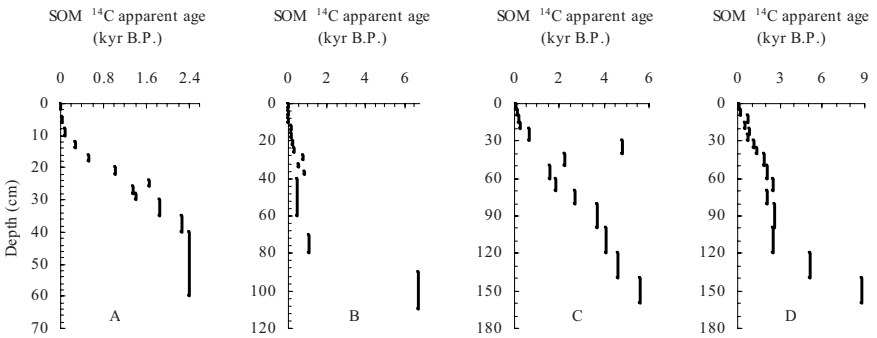

Fig. 5. Variations of SOM ^{14}C apparent ages with depth for the soil profiles with different elevations at the Dinghushan Biosphere Reserve (**A**: GC Profile, **B**: SL Profile, **C**: WKS profile, **D**: QYS profile).

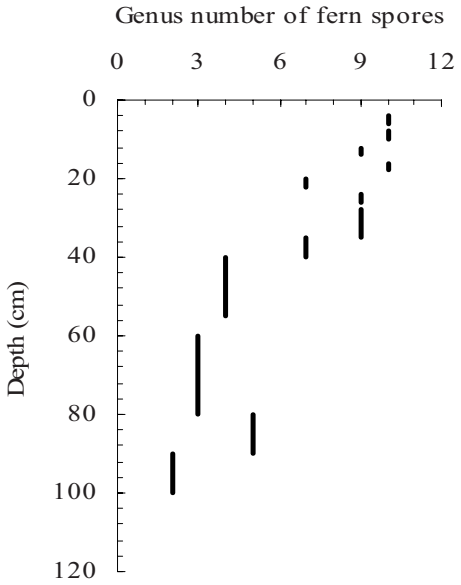

Fig. 6. Variations of genus number of fern spores with depth for SL profile at the Dinghushan Biosphere Reserve.

4 Discussion

4.1 Variations in SOM $\delta^{13}C$ Value and SOC Concentration with Depth

Rapid turnover of SOM usually occurs within 100 years, and SOM decomposes with SOC concentration decreasing rapidly with depth from the surface (Figs. 4, 5) and SOM $\delta^{13}C$ values becoming enriched in ^{13}C rapidly due to carbon isotope fractionation (Fig. 1). After 200–300 years, most of the labile SOM have decomposed, resulting in the greatest $\delta^{13}C$ values generally (Figs. 1, 5). SOC concentrations then reduce slightly with depth (Fig. 4), and SOM $\delta^{13}C$ values become depleted in ^{13}C gradually with depth due to the decomposition of SOM compartments with high $\delta^{13}C$ values (Fig. 1). After about 1500–1800 years, SOC concentrations reach their lowest levels generally (Figs. 4, 5), and SOM $\delta^{13}C$ values become stable (Fig. 1), indicating that the SOM is mainly of stable compartment in the deep soil.

There have been many proposed explanations to describe the enrichment of SOM $\delta^{13}C$ in ^{13}C with depth in soil profiles (Powers and Schlesinger 2002). Based on above analyses, variations of SOM $\delta^{13}C$ with depth were attributed mainly to isotope fractionation due to SOM decomposition at the DHSBR. This is consistent with our early study (Chen et al. 2002a) and other studies in different climatic zones (Balesdent et al. 1993; Ågren et al. 1996; Schweizer et al. 1999; Powers and Schlesinger 2002; Hobbie et al. 2004).

SOM $\delta^{13}C$ vs depth curves differ between profiles with different elevations at the DHSBR (Fig. 1), probably due to differences in factors controlling soil development, such as, topography and vegetation, at different elevations (Table 1). This phenomenon had also been identified at the Xiaoliang Ecological Station, CAS (Chen et al. 2002a), based on comparisons of SOM $\delta^{13}C$ vs depth curves between soil profiles with different restoration histories of above-ground vegetation. SOM $\delta^{13}C$ vs depth curve may be used as a useful proxy for studying soil carbon dynamics based on comparison analyses.

Table 4. Sporopollen abundance in the soil samples of SL profile at the Dinghushan Biosphere Reserve, South China

Sample number	Depth (m)	Abundance of pollen (grain g^{-1})	Total abundance of sporopollen (grain g^{-1})
SL-03	0.04–0.06	2462	7351
SL-05	0.08–0.1	610	2892
SL-07	0.12–0.14	315	3359
SL-09	0.16–0.18	6	524
SL-11	0.2–0.22	2	389
SL-13	0.24–0.26	1	300
SL-15	0.28–0.3	0	125
SL-16	0.3–0.35	0	55
SL-17	0.35–0.4	0	79
SL-18	0.4–0.45	0	12
SL-19	0.45–0.5	0	57
SL-20	0.5–0.55	0	33
SL-22	0.6–0.7	0	21
SL-23	0.7–0.8	0	31
SL-24	0.8–0.9	0	52
SL-25	0.9–1.1	0	4

The depths with the maximal SOM $\delta^{13}C$ value of the studied soil profiles show an increasing tendency with the decreasing of elevation (Fig. 2), which is related well to the increasing thickness of soil profile with decreasing of elevation. Variations of SOM $\delta^{13}C$ with depth correspond well with those of SOC concentration with depth (Chen et al. 2002a). It is then inferred that the depth with the maximal SOM $\delta^{13}C$ value is controlled mainly by soil development and SOM composition, and is governed indirectly by topography and above-ground vegetation. This is presumably the

site-related characteristic for variations of SOM $\delta^{13}C$ with depth at the DHSBR, as reported for a temperate forest in Balesdent et al. (1993).

The discrepancy between SOM $\delta^{13}C$ of topsoil and the maximal SOM $\delta^{13}C$ is not consistent between the soil profiles at different elevations (Tables 1, 3), suggesting that there existed discrepancies in both SOM composition and SOM turnover between the studied soil profiles at the DHSBR. The discrepancy of SOM $\delta^{13}C$ (Table 3) presumably indicates the intensity of carbon isotope fractionation during SOM decomposition. The larger discrepancy probably indicates greater intensity of carbon isotope fractionation and higher extent of SOM decomposition.

4.2 $\delta^{13}C$ Values of Topsoil SOM and Above-Ground Plant Debris

SOM $\delta^{13}C$ of topsoil become enriched in ^{13}C with elevation at the DHSBR (Fig. 3), suggesting that the above-ground vegetations turn gradually with elevation from those being dominated by C3 species to those being dominated by C4 species. This is consistent with the vertical distribution patterns of vegetation at the DHSBR (Table 1). SOM $\delta^{13}C$ value of topsoil is not the minimal for JLS profile (Figs. 1), and is greater than the surface values at other sites (Figs. 1, 3). The $\delta^{13}C$ value of plant debris is evidently greater than that of the SOM in topsoil of JLS profile (Fig. 3), presumably because the plant debris samples were mainly of herbaceous (Table 1), and moss and weeds attaching on the ground were neglected during sample collection.

SOM $\delta^{13}C$ of topsoil is greater than $\delta^{13}C$ of plant debris at the DHSBR (Fig. 3), indicating that stable isotopes fractionation occurred during formation of SOM from decomposition of organic materials (OM) in plant debris. The SOM in topsoil originates mainly from plant debris of the above-ground vegetation (Kononova 1966; Van Cleve and Powers 1995). Hobbie et al. (2004) reported that $\delta^{13}C$ of new A horizon carbon was about 4‰ enriched in ^{13}C relative to that of foliage. Except for the plant debris $\delta^{13}C$ of GC profile, both SOM $\delta^{13}C$ of topsoil and plant debris $\delta^{13}C$ show similar pattern in variations with elevation at the DHSBR (Fig. 3), indicating transformation of OM from plant debris into the SOM in topsoil.

The increments in SOM $\delta^{13}C$ of topsoil relative to $\delta^{13}C$ of plant debris do not show regular variations with elevation at the DHSBR (Fig. 3), which is presumably due to the discrepancy in turnover of plant debris between different species of vegetation. Different plant species are known to produce organic matter compounds that vary in abundance and nature as a function of species (Grayston et al. 1996).

SOM $\Delta^{14}C$ values are generally positive in the upper soil layers, due to entrance of "bomb ^{14}C" into these layers through decomposition of plant debris and fine roots (Shen et al. 1999). The greater value of SOM $\Delta^{14}C$ (>0) of topsoil indicates rapid entrance of "bomb ^{14}C" into topsoil from plant debris, due to the greater SOM turnover rate of topsoil. Whereas, the less value of SOM $\Delta^{14}C$ (>0) of topsoil suggests slow entrance of "bomb ^{14}C" into topsoil from plant debris, due to the less SOM turnover rate of topsoil.

The increment in SOM $\delta^{13}C$ of topsoil relative to $\delta^{13}C$ of plant debris correlates well with SOM $\Delta^{14}C$ of topsoil (Fig. 7). The greater the SOM turnover rate of topsoil, the shorter period of time when OM is absorbed into SOM through decomposition of plant debris. Stable carbon isotope fractionation accomplishes then in a shorter period of time, so the increment in SOM $\delta^{13}C$ of topsoil relative to $\delta^{13}C$ of plant debris is greater than that when stable carbon isotope fractionation accomplishes in a longer period of time due to slow SOM turnover in topsoil. The increment in SOM $\delta^{13}C$ of topsoil relative to $\delta^{13}C$ of plant debris is then controlled mainly by SOM turnover.

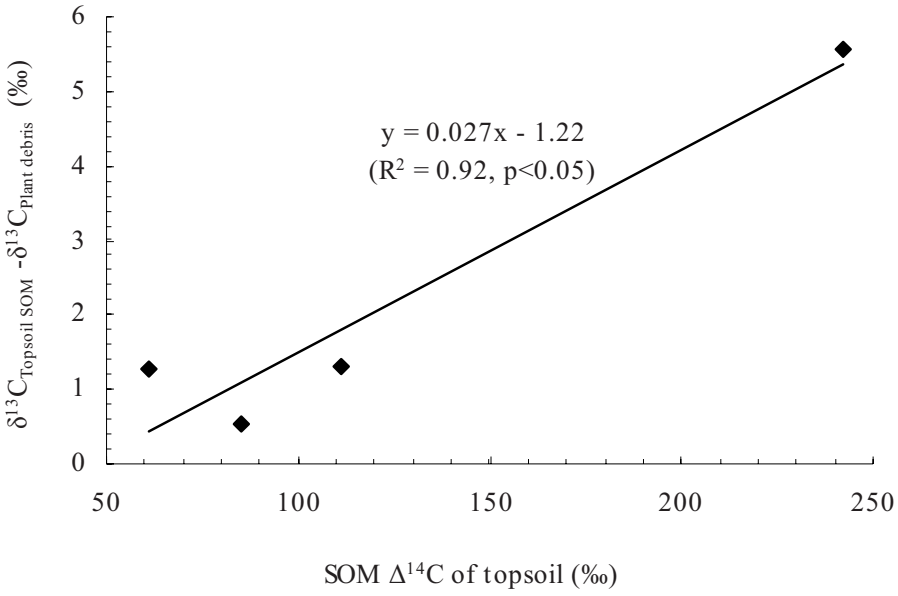

Fig. 7. Relationship between topsoil SOM $\Delta^{14}C$ and ($\delta^{13}C_{Topsoil\ SOM} - \delta^{13}C_{Plant\ debris}$) of the soil profiles with different elevations at the Dinghushan Biosphere Reserve (Acronyms: SOM $\delta^{13}C$ of topsoil $\delta^{13}C_{Topsoil\ SOM}$, $\delta^{13}C$ of plant debris $\delta^{13}C_{Plant\ debris}$).

4.3 SOM ^{14}C Apparent Ages

The increasing tendency of SOM ^{14}C apparent ages with depth for the soil profiles with different elevations (Fig. 5) suggests that the development of the soil profiles at the DHSBR presumably followed the model that deposition and pedogenesis occurring alternatively. The soil profiles were thickened by deposition, and then the deposited materials experienced pedogenesis and turned to be soils. The former soil layers were buried with deposition of new materials, and soil thickened gradually and SOM ^{14}C apparent ages then show increasing tendency with depth.

SOM ^{14}C apparent ages of some soil layers are occasionally older than those of the underlying soils (Fig. 5), which is probably due to the impacts of biological activities on distribution of SOM with depth. Some insects or animals, for example, ant and earthworm, might bring soil with old carbon upwards from the deep section. SOM ^{14}C apparent ages of the deep boundaries of the studied soil profiles do not show clear correlation with elevation (Tables 1, 3). The beginning of soil development was then not synchronous for the studied soil profiles at the DHSBR, showing potential controls of topography on soil evolution.

The SOM ^{14}C apparent age of soil section (0–0.05 m) is 80 yr B.P. for WKS profile and 140 yr B.P. for QYS profile, suggesting that the upper soil layers had been contaminated by some old carbons with negative $\Delta^{14}C$ values. Contaminations from old carbon and "bomb ^{14}C" can result in obvious alterations in SOM $\Delta^{14}C$ values. However, SOM $\delta^{13}C$ value is relatively less susceptible to such contaminations, because the abundance of ^{13}C is much greater than that of ^{14}C in nature system. This indicates that ^{14}C analysis alone is not adequate for studying SOM dynamics.

The "bomb ^{14}C" penetrating depth relates directly to the impacts of modern vegetations and microbes on soil development (Shen et al. 1999), and is then the quantitative indicator for the impacts of modern biologic activities on soil profile. The magnitude of this depth is also governed by the properties of soil profiles (Chen et al. 2002b), showing influence of local topography on distribution of SOM ^{14}C with depth. Although the "bomb ^{14}C" penetrating depth and the depth with the maximal SOM $\delta^{13}C$ value differed in magnitude and origin, they both indicate controls of topography and vegetation on distributions of SOM and carbon isotopes with depth.

The regular vertical distribution of SOM compartments with different turnover rates (Chen et al. 2002b) and the exponential reduction of SOC concentration with depth (Fig. 4) presumably both resulted from regular decomposition of different SOM compartments during soil development at the DHSBR. SOM turnover can result in temporal variations in SOM $\Delta^{14}C$

and SOM $\delta^{13}C$ values, which is the rationale for studies on SOM turnover and mechanism of soil development based on carbon isotopic tracing.

4.4 Vertical Distributions of Pollen and Spores

Pollen were found only in the sections above 0.26 m (Table 4), indicating that pollen were not prone to be leached after deposition. No pollen was found in the low part of SL profile, suggesting that pollen had probably been destroyed by the intense weathering during pedogenesis. Fern distributes extensively in the monsoonal area of South China, its development (sporophyte and gametophyte) depends mainly on cycling of above-ground water, and precipitation exerts great impacts on transportation and penetration of spores into soil (Zheng et al. 2002). The fern spores occurred below 0.26 m resulted presumably from transportation of spores downwards with penetration of above-ground water.

The soil was loose and contained abundant spores with more genera above 0.4 m (Table 4, Fig. 6), and became denser due to the abrupt increasing in clay concentration below 0.4 m of SL profile (Chen et al. 2002a), which restrained the penetration of spores with leaching of soil water. Consequently, the quantity of spores decreased with depth (Table 4) and the genus became monotonous (Fig. 6). The loose/stiff quality of soil is then a critical factor controlling the penetration of spores.

The regular distributions of pollen and spores with depth of SL profile indicate that weathering was intense and leaching due to precipitation was evident during soil development. SOM ^{14}C apparent ages increase with depth (Fig. 5), suggesting that SOM in the low section had not been contaminated heavily by leaching of SOM from the above section. SOM was then not prone to be leached as were the spores during soil development. Although the effect of eluviation on the vertical distribution of fern spores is marked, and eluviation is a main process for the loss of dissolved OM from soil, its impact on the vertical distribution of SOM is actually very limited.

5 Conclusions

The variations in SOC concentration and SOM $\delta^{13}C$ value with depth show both consistent tendencies for soil profiles with different elevations at the DHSBR. This was mainly attributed to the regular decomposition of SOM compartments with different turnover rates during soil development.

Carbon isotope fractionation due to SOM turnover can result in considerable enrichment of SOM $\delta^{13}C$ value in ^{13}C.

Vegetation and topography were main factors controlling the distributions of SOM and carbon isotopes with depth at the DHSBR. The SOM $\delta^{13}C$ of topsoil and plant debris $\delta^{13}C$ became both enriched in ^{13}C with elevation, showing the regular variations of vegetation with elevation and the transformation of OM from plant debris into SOM in topsoil. The SOM $\delta^{13}C$ of topsoil can be used as a reliable proxy for studying vertical distribution of vegetation in a mountainous region.

SOM was not prone to be leached as were the spores during soil development at the DHSBR. The development of soil at the DHSBR followed the model that deposition and pedogenesis occurring alternatively. The distribution of SOM with depth is governed directly by input and SOM turnover during soil evolution, and indirectly by factors controlling soil development, such as vegetation and topography.

Acknowledgements

We thank Professor Z.Y. Yu and Dr. H. Ren of the South China Institute of Botany, CAS, for their valuable suggestions for field work. We also thank Dr. Y.C. Gao and Dr. W.J. Shen of the South China Institute of Botany and Dr. Y. Yang of the Guangzhou Institute of Geochemistry, CAS, for their help in field sampling. We are indebted to Dr. Z. Zheng of Zhongshan University, for his kind helps in sporopollen analysis. We thank the two anonymous referees for their valuable suggestions to improve the manuscript. The work was supported by the National Natural Science Foundation of China (Grant Nos. 39728102, 40202032), the China Postdoctoral Science Foundation, the Natural Science Foundation of Guangdong Province (Grant no. 984105), the Science Foundation of Shanghai Educational Committee for young teachers, and Heshan Field Station for Integrated Investigation, CAS.

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10 Bioavailability of Soil-Sorbed Pesticides and Organic Contaminants

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1 Introduction

Toxic organic chemicals find their way into our environment as a result of industrial and agricultural activities. There are at least 75,000 toxic chemicals in common use worldwide (US EPA 2005). In the U.S., about 2.2 billion kilograms of chemicals are used as pesticides each year, with agricultural usage accounting for 77% (Kiely et al. 2004). Many pesticides and other organic chemicals have been found to accumulate in nature because the release rates exceed the rates of microbial and chemical degradation. Low biodegradation rates can be attributed to: (1) limited biochemical potentials, i.e., chemicals with structures foreign to nature are less likely to be degraded without a long adaptation period; and (2) limited bioavailability of contaminants or other substances (e.g. electron acceptors) to microorganisms. Our understanding of pesticide bioavailability has important ramifications

for environmental fate modeling, risk assessment and remediation. Limited bioavailability may lead to unexpected chemical persistence in soils hence increasing the likelihood of ground- or surface-water contamination (Pignatello et al. 1987). In the remediation of contaminated soils, bioavailability affects the clean-up time, cost, and the end-point of the process. Bioavailability of pesticides and other organic contaminants has been identified as a major limitation to complete bioremediation of contaminated soils (US EPA 1999).

2 General Concept of Bioavailability

The term "bioavailability" has different meanings in different contexts and disciplines. Numerous definitions of bioavailability exist. Research on the relationship between bioavailability and chemical speciation (forms) originated in the field of soil fertility in the search for a good predictor for the bioavailability of essential plant nutrients (Traina and Laperche 1999). It is well accepted that dissolved nutrients are more labile and bioavailable to plants than solid-phase forms (including sorbed species). The same has been considered to be true for organic contaminants and their availability for microbial degradation.

Biological availability of environmental pollutants in ecotoxicology context was defined at a National Science Foundation workshop on ecosystem processes and organic contaminants held in 1975, as "the extent to which a toxic contaminant is available for biologically mediated transformations and/or biological actions in an aquatic environment" (Dickson et al. 1994). From biodegradation point of view, bioavailability was defined as the accessibility of a chemical for assimilation by microorganisms (Alexander 2000). Bosma et al. (1997) defined bioavailability as the ratio of the capacity of an organism's environment to transport the chemical to the capacity of the organism to degrade that chemical. There are many more versions of definitions for bioavailability. Overall, bioavailability has been defined "in either relative or absolute terms and in either chemical or biological terms" (Dickson et al. 1994). There is no single universally accepted definition for all situations.

The Committee on Bioavailability of Contaminants in Soils and Sediments of National Research Council (NRC) of the National Academies USA chose to define *the bioavailability process* instead of bioavailability to avoid the confounding use of the term bioavailability (National Research Council 2003). According to the NRC report, *bioavailability processes* are "the individual physical, chemical, and biological interactions

that determine the exposure of organisms to chemicals associated with soils and sediments". A scheme of bioavailability processes is shown in Fig. 1.

Bioavailability issues have been reviewed previously (Mihelcic et al. 1993; Boesten 1993; Baveye and Bladon 1999; Ehlers and Luthy 2003). In this review, we discuss specifically the bioavailability of soil- or sediment-sorbed organic contaminants to pollutant-degrading bacteria. Direct uptake of sorbed contaminants is perhaps the most controversial and least understood process. The definition of bioavailability given by Alexander (2000) will be used in this review. The term "bioaccessibility" encompasses what is immediately available plus that which may become available, whereas bioavailability refers to what is available immediately.

3 Assessing Bioavailability of Soil-Sorbed Chemicals Experimentally

Significant amounts of organic contaminants released to the environment become sorbed to soil and sediment. Soil clay minerals and organic matter are the primary sorptive compartments for these contaminants. Generally, soil-sorbed organic contaminants and pesticides have been considered unavailable for biodegradation. Numerous reports provided supporting evidence (Steen et al. 1980; Ogram et al. 1985; Shimp and Young 1988; Smith et al. 1992). Most mathematical models for coupled sorption and biodegradation processes have been developed with the assumption that only solution-phase substrate was subject to biodegradation (Brusseau et al. 1992; Sabljic 1989; Scow and Johnson 1997). However, some evidence suggests that sorbed contaminants can be degraded by microorganisms, or at least that desorption into bulk solution is not a prerequisite for biodegradation. For sorbed contaminants to become bioavailable, there are two scenarios. As illustrated in Fig. 1, scenario one involves desorption of sorbed contaminant into soil solution and subsequent uptake by bacterial cells (indicated by letters A and B). In scenario two, the sorbed compound partitions into bacterial cells without prior desorption into the bulk soil solution although the compound may desorb into the microscopic environment between the cell and the surface of soil particle (indicated by letter C in Fig. 1).

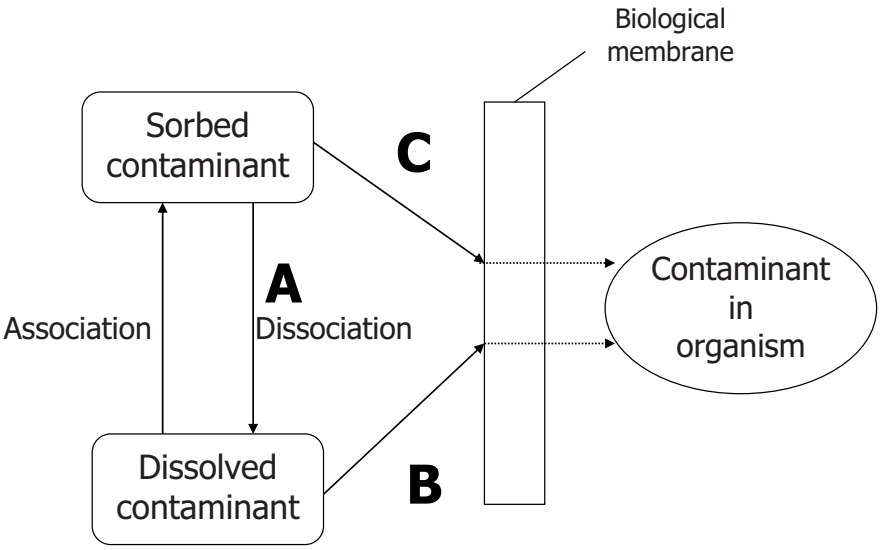

Fig. 1. A schematic of bioavailability processes in soil or sediment (modified after National Research Council 2003). Letters **A** and **B** indicate desorption of sorbed contaminant and subsequent uptake by bacterial cells, respectively. Letter **C** indicates partition of sorbed contaminant into bacterial cells without prior desorption into the bulk soil solution. Reprinted with permission from National Research Council (2003). Copyright (2003) the National Academy of Sciences, courtesy of the National Academies Press, Washington, D.C.

To evaluate the bioavailability of soil-sorbed contaminants, Guerin and Boyd (1992) designed a kinetic mineralization assay using a batch system. A series of soil-free controls and soil slurries containing radioactive labeled and nonlabeled chemicals at appropriate concentrations were set up in sealed serum bottles. Once the sorption equilibrium was reached, the serum bottles were inoculated with bacteria that are capable of degrading the target chemical. At various time intervals, equal volume of aqueous solution and headspace were sampled to monitor $^{14}CO_2$ production. According to Michaelis-Menten kinetics,

$$V = \frac{V_{\max} \bullet S}{K_m + S},$$

at substrate concentrations (S) below the Michaelis-Menten constant, K_m (equal to the substrate concentration at half of the maximum reaction

rate, V_{max}), the mineralization rates of resting (non-growing) cells are linearly proportional to substrate concentrations. First-order mineralization rates can be obtained by plotting percent of substrate mineralization (P) as a function of incubation time: $P = P_{max} (1 - e^{-kt})$, where k is the first-order rate constant (min^{-1}) and P_{max} is the maximal percent mineralized. P_{max} and k were estimated by nonlinear regression analysis and used to calculate the initial mineralization rate (IMR): IMR ($\mu g\ L^{-1}min^{-1}$) = $k \bullet P_{max} \bullet S$. The plot of IMR vs. equilibrium aqueous phase concentration is linear (Fig. 2). The equilibrium aqueous phase concentrations can be determined using sorption isotherms. If sorbed chemical is unavailable to bacteria, i.e., only chemicals in the aqueous phase can be degraded in soil slurries, IMR values of soil slurries should be equal to the control rate and be coincidental with the soil-free line. If the measured IMR in soil slurries are above the soil-free control line (Fig. 2), it indicates that bacteria have access to sorbed chemical or that desorption is rapid relative to degradation and cells experience a higher substrate concentration than that in the solution. Guerin and Boyd (1992) used this approach to probe the bioavailability of soil-sorbed naphthalene. Bioavailability assays were performed using two naphthalene-degrading bacteria, *Pseudomonas putida* ATCC 17484 and *Alcaligenes* sp. strain NP-Alk, and four surface soils. For strain NP-Alk, the IMRs in soil slurries fell very close to the soil-free control line (Fig. 3). This suggests the unavailability of the sorbed pool of naphthalene and the hypothesis that sorbed substrate is unavailable was valid. For ATCC 17484, upward deviations of IMR values from the soil-free control line were observed (Fig. 3), indicating that this organism had direct access to sorbed phase napthalene. It seems that whether or not sorbed organic contaminants are bioavailable depends on organism-specific properties and physiochemical factors. Generalizations regarding the bioavailability of sorbed chemicals need reexamination.

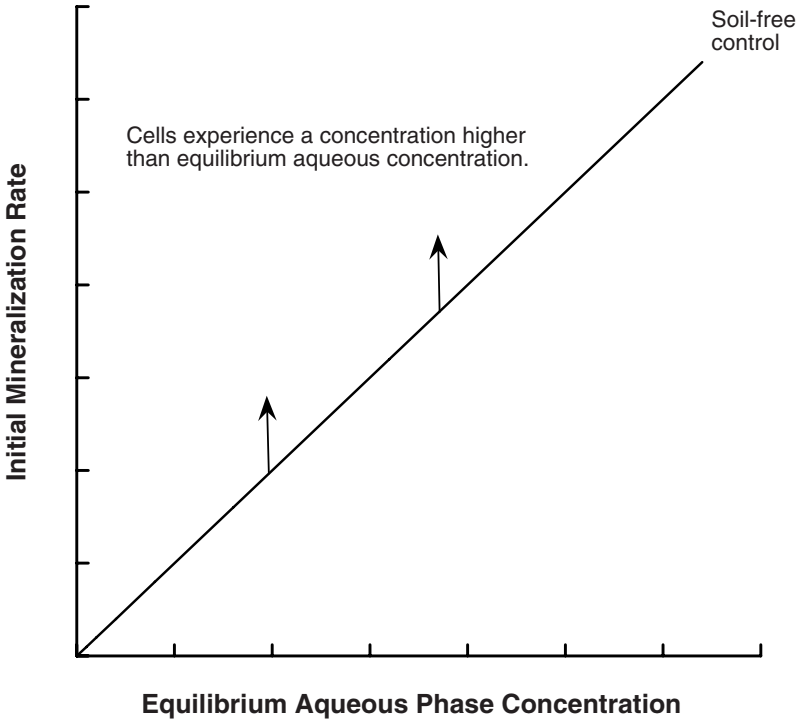

Fig. 2. A diagram illustrating the presentation and interpretation of data obtained from a bioavailability assay developed by Guerin and Boyd (1992).

To further understand this phenomenon, bioavailability assays were conducted using two biphenyl-degrading bacteria, *Pseudomonas putida* strain P106 and *Rhodococcus erythropolis* strain NY05, and four soils of different organic carbon contents (Feng et al. 2000). In these systems the total mass of biphenyl was constant (equivalent to the 400 μg L^{-1} soil-free control) but the concentration in solution varied depending on the degree of sorption, which in turn depended on the specific soil. For example, with the Colwood soil the aqueous biphenyl concentration was reduced from

400 to 20 μg L^{-1} due to sorption. If only aqueous phase, but not sorbed phase, biphenyl was available, then the measured IMR in the soil slurry should coincide with the control line (assuming no contribution from biphenyl desorption). As shown in Fig. 4 the rates in soil slurries were much greater than expected on the basis of solution phase concentrations. In fact the rates in all four soil slurry systems were essentially equivalent to those in the soil-free controls with the same total mass of biphenyl. Remarkably, reducing the aqueous phase biphenyl concentration as much as 20-fold due to sorption did not manifest any diminution in the IMRs. These data strongly indicate the ability of strain P106 to access the pool of sorbed biphenyl. Soil-sorbed biphenyl also appeared available to strain NY05, albeit to a lesser extent.

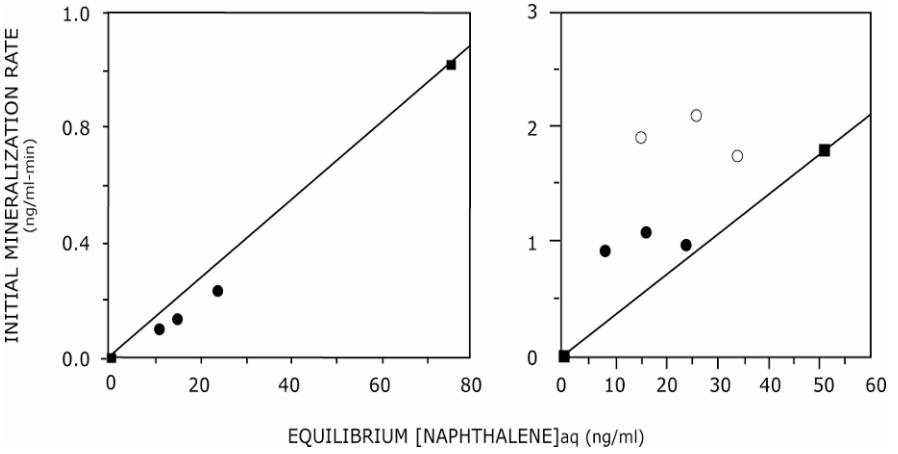

Fig. 3. Plots of initial mineralization rates (IMR) versus equilibrium aqueous phase concentrations for naphthalene-degrading bacteria. *Open circles* represent Capac soil, *closed circles* represent Colwood soil, and *squares* represent soil-free control data points. Reprinted with permission from Guerin and Boyd (1992). Copyright (1992) American Society for Microbiology.

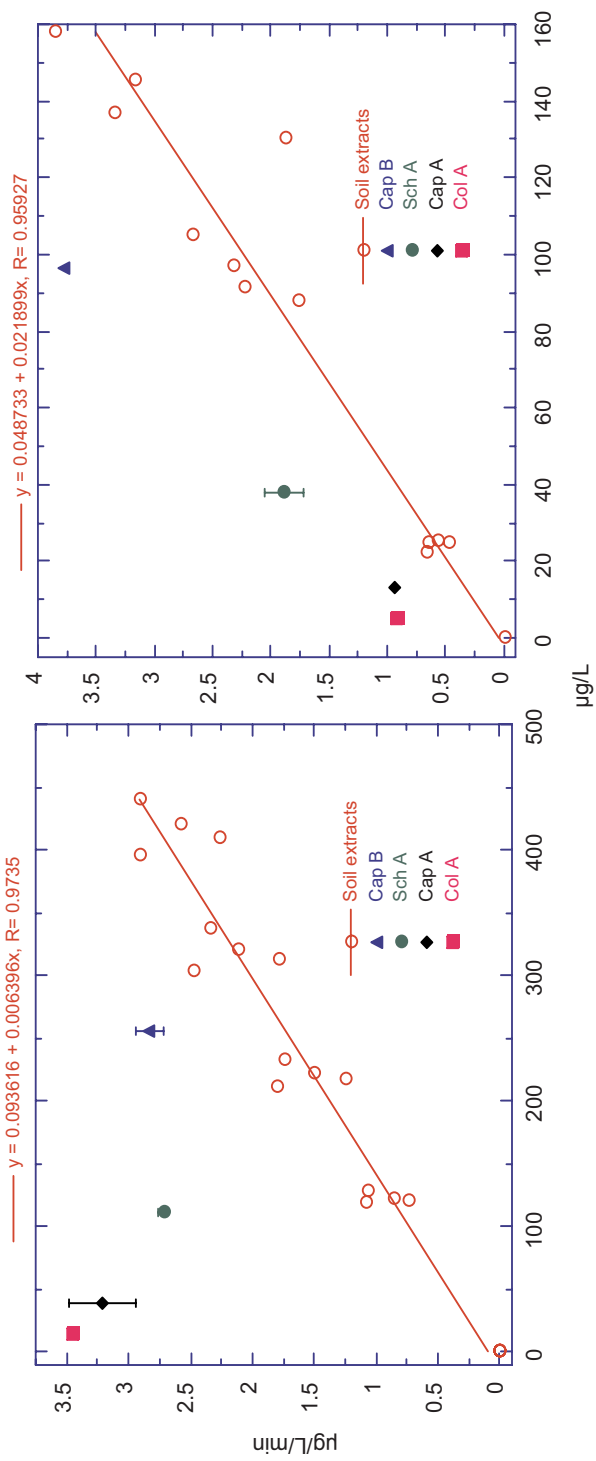

Fig. 4. Plots of initial mineralization rates (IMR) versus equilibrium aqueous phase concentrations for biphenyl-degrading bacteria. Cap B, Sch A, Cap A, and Col A indicate soils used in the study. Letters A and B indicate A and B soil horizon, respectively. Cap, Sch, and Col represent Capac, Schoolcraft, and Colwood soil, respectively. Reprinted with permission from Feng et al. (2000). Copyright (2000) American Chemical Society.

One limitation of the batch mineralization methodology is that it does not provide a quantitative description of the dynamics of contaminant sorption/desorption and its subsequent effect on biodegradation. In an attempt to strengthen the interpretation that strain P106 had immediate and nearly complete access to the pool of sorbed biphenyl, a coupled desorption/biodegradation model which accounted for the contribution of desorption to biodegradation was developed (Feng et al. 2000). Without information regarding desorption kinetics, this model only defined the limiting cases of instantaneous desorption and no desorption. The measured biphenyl mineralization curves for P106 fell well above the defined case CO_2 production curves where desorption was assumed *instantaneous* (Fig. 5). This provided further evidence for the ability of P106 to access sorbed biphenyl.

In subsequent studies, a more sophisticated mathematical model coupled with measurements of biodegradation/mineralization and desorption kinetics was developed to more completely account for the effects of sorption/desorption processes on bioavailability. In this study, three atrazine-degrading bacteria (*Pseudomonas* sp. strain ADP, *Agrobacterium radiobacter* strain J14a and *Ralstonia* sp. strain M91-3), five soils, and K-montmorillonte were used in the bioavailability assays (Park et al. 2003). In eleven out of eighteen cases, the mineralization of atrazine was accurately predicted by the Desorption-Biodegradation-Mineralization (DBM) model, which accounts for the extents and rates of sorption/desorption processes and assumes biodegradation of liquid-phase, but not sorbed atrazine. However, for the Houghton muck soil with all three bacteria and *Pseudomonas* sp. strain ADP with Colwood and Hartsells soils, mineralization rates greater than those expected on the basis of aqueous-phase atrazine concentration were observed. Even the assumption of instantaneous desorption could not account for the elevated rates in the Houghton muck soil, which manifested the highest sorbed atrazine concentrations. This may be explained by that bacteria access the localized regions where atrazine is sorbed and that the concentrations found support higher mineralization rates than predicted on the basis of aqueous-phase concentrations. Calvillo and Alexander (1996) attempted to quantify the effects of desorption on mineralization of sorbed chemicals and showed that mineralization rates of biphenyl sorbed to polyacrylic beads by a microbial consortium were higher than the biphenyl desorption rates. They isolated two pure cultures of bacteria from this consortium and found that individually these two isolates mineralized biphenyl in solution but not sorbed substrate. However, combination of the two isolates resulted in utilization of sorbed biphenyl. The authors suggested that the sorbed chemicals were directly

available to bacteria without direct dissolution (Calvillo and Alexander 1996; Tang et al. 1998).

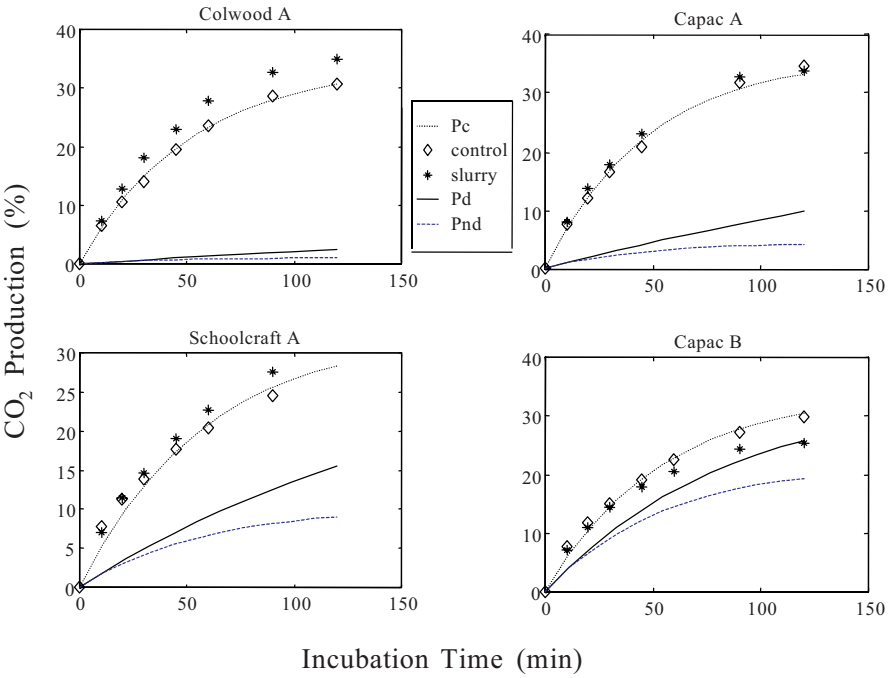

Fig. 5. Mineralization of biphenyl by *Pseudomonas* strain P106 in four soil-free controls and soil slurries. Soil slurries contained 0.1 μCi ^{14}C-labeled and ~400 μg L^{-1} unlabeled biphenyl initially. P_c is estimated line from the soil-free control data. P_d is the predicted mineralization curve assuming instantaneous desorption. P_{nd} is the predicted mineralization curve assuming no desorption. Reprinted with permission from Feng et al. (2000). Copyright (2000) American Chemical Society.

Recognizing the limitations of the traditional enrichment system, several researchers designed untraditional enrichment techniques to isolate bacteria capable of degrading sorbed organic chemicals (Calvillo and Alexander 1996; Tang et al. 1998). Calvillo and Alexander (1996) provided phenanthrene sorbed to polyacrylic beads (SM-7 Biobeads) instead of dissolved substrate in the enrichment cultures. A bacterium obtained by enrichment on sorbed substrate mineralized phenanthrene sorbed to beads or a lake-bottom sediment, whereas a bacterium obtained by enrichment on non-sorbed phenanthrene did not. Bastiaens et al. (2000) compared polycyclic aromatic hydrocarbon (PAH)-utilizing bacteria isolated from PAH-contaminated soil and sludge samples using two different enrichment

procedures, i.e., PAHs supplied as crystals in liquid mineral medium vs PAHs supplied in sorbed form on hydrophobic membranes. Both procedures were successful in obtaining PAH-degrading bacteria, but selected different bacterial strains. The liquid enrichment mainly selected for *Sphingomonas* sp., whereas the membrane method selected for *Mycobacterium* sp. exclusively. The new *Mycobacterium* isolates were strongly hydrophobic and adhered strongly to different surfaces "which might be useful to biodegrade sorbed PAHs in soils and sludge" (Bastiaens et al. 2000). Grosser et al. (2000) attempted to isolate phenanthrene-degrading bacteria using different model sorbents of varying phenanthrene sorptive capacities in their enrichment system. These sorbents were Amberlite carboxylic acid cation-exchange resin, SM-7 Biobeads (polyacrylic resin), and SM-2 Biobeads (divinyl benzene resin). More than 40 phenanthrene-degrading bacterial isolates were cultivated and individual isolates showed significant variation in their ability to mineralize phenanthrene sorbed to solid phases. Although an inverse relationship between the phenanthrene degradation rates and phenanthrene sorption to model sorbents was observed, the degradation rates in the presence of Amberlite resin and SM-7 Biobeads were much higher than those predicted from the phenanthrene desorption rates. In the accompanying paper, Friedrich et al. (2000) reported that the denaturing gradient gel electrophoresis (DGGE) patterns obtained from enrichment cultures containing sand or no sorbents were different from those obtained from enrichment cultures containing phenanthrene sorbed to Amberlite resin and SM-7 Biobeads. SM-7 Biobeads enrichment selected for mycobacterial phenanthrene mineralizers, whereas Amberlite resin selected for a *Burkholderia* sp. These results suggest that different phenanthrene-degrading bacteria adapt to different phenanthrene bioavailability. In another study, Vacca et al. (2005) carried out paired enrichments to compare phenanthrene degraders isolated using a traditional technique with non-sorbed phenanthrene to those using an enrichment system with phenanthrene sorbed by humic acids. This study showed that only isolates obtained from enrichments with humic acid-sorbed phenanthrene were capable of mineralizing sorbed phenanthrene at levels far above those which desorption and initial aqueous phase phenanthrene could sustain. Thus, some or all of the humic acid-sorbed phenanthrene was available for uptake without requiring desorption.

4 Factors Affecting Bioavailability of Soil-Sorbed Chemicals

Bioavailability of soil-sorbed organic chemicals depends on a variety of physicochemical factors as well as the characteristics of microorganisms involved. Although many studies have provided insight to our understanding of the bioavailability issue, simple correlations have not been found between bioavailability and physicochemical properties of sorbed chemicals or sorbents, nor microbial characteristics.

4.1 Nature of the Chemical

One factor to consider is the nature of the chemical compound. Organic contaminants differ in chemical structure, reactivity, and many other physical and chemical properties. Some are recalcitrant whereas others are subject to biodegradation to different extents. Most research on microbial metabolism of organic chemicals concentrates on water-soluble molecules or water-soluble fraction of a compound. Polarity or hydrophilicity of a biodegradable compound is considered to be an important characteristic influencing sorption and therefore biodegradation (Barriuso et al. 2004). Sorption of many nonpolar pesticides and organic contaminants are strongly correlated with soil organic matter contents and the compound's hydrophobicity (e.g. Lambert 1967, 1968; Chiou et al. 1979; Briggs 1981; Chiou et al. 1983; Chiou 2002). Retention of these contaminants in soils can be predicted using organic carbon (OC) normalized sorption coefficients (K_{oc}), which can be estimated using the octanol-water partition coefficients (K_{ow}) or inverse of water solubility (e.g. Karickhoff 1981). In general, the more polar a compound, the less important is hydrophobic partitioning. Polar and semi-polar organic compounds may also interact with mineral surfaces (Laird et al. 1992; Laird and Fleming 1999; Sheng and Boyd 2000; Boyd et al. 2001). Sorption of ionizable organic compounds, however, is more dependent on the surrounding solution chemistry than nonpolar compounds. Graber and Borisover (2005) indicated that compound solvation interactions in the bulk aqueous phase may mask sorbate – SOM interaction for different organic compounds when K_{oc}-based approach is used. They used a thermodynamic cycle approach by eliminating the contribution from compound hydration in the aqueous phase to examine the interaction between natural organic matter and organic compounds. They concluded that polar organic compounds interacted much stronger with SOM than aromatic hydrocarbons and their halogen-substituted derivatives of the same electronic polarizability. Since the nature of a compound influences sorption capacity and the strength of

interaction, it is likely to affect bioavailability as well. However, currently no general trend has been observed between compound polarity and bioavailability. Organic compounds that have been evaluated in terms of their availability to bacteria include various pesticides (e.g. 2,4-D and atrazine) and PAHs (e.g. naphthalene and phenanthrene).

4.2 Nature of the Sorbent

Another physicochemical factor to consider is the nature of the sorbent itself. Soil organic matter content, mineral composition, and particle size distribution all affect sorption/desorption processes and thus contaminant bioavailability. Several studies have been conducted to examine how SOM contents affect bioavailability of organic contaminants. Guerin and Boyd (1993) observed that naphthalene sorbed to high OC soils was less available to *P. putida* strain 17484 than that sorbed to low OC soils. It was suggested that a larger portion of naphthalene was present in a nonlabile phase in a high OC soil, and therefore naphthalene was less accessible to potential microbial degraders compared with that in a low OC soil. White et al. (1997) examined the sequestration and bioavailability of phenanthrene in seven soils with SOM contents ranging from 1.1 to 13%. They did not find an apparent relationship between soil organic matter content and mineralization of phenanthrene. Chung and Alexander (1998) also reached the same conclusion after investigating bioavailability of atrazine and phenanthrene in 16 dissimilar soils. However, further analysis by Chung and Alexander (2002) using the same data set showed that some but not all measures of phenanthrene and atrazine sequestration were correlated with OC content, nanoporosity, or CEC. Feng et al. (2000) found that biphenyl sorbed to high OC soils was less available to biphenyl-degrading *R. erythropolis* NY05 but not to *P. putida* P106, than that sorbed to low OC soils. In another study, Park et al. (2003) found that atrazine sorbed by an organic soil (38% OC) was available to all three atrazine-degrading bacteria tested whereas atrazine sorbed by mineral soils was not available to these organisms in most cases. At the present time, it is not possible to generalize the effect of soil organic matter on bioavailability of organic contaminants.

In addition to SOM, clay minerals are another important component that may influence contaminant-soil interactions. Expandable 2:1 type clays are usually more reactive than other clay minerals. Park et al. (2003) used a K-saturated montmorillonite as a sorbent to evaluate the availability of sorbed atrazine to three atrazine-degrading bacteria. K-saturated montmorillonite has a high atrazine sorption capacity with a Freundlich sorption

coefficient of 43.8 (mg kg^{-1})/(mg L^{-1})^n. The clay caused inhibition of atrazine mineralization for two organisms. This may have resulted from clay particles coating the bacterial cells since the fine clay particles were substantially smaller than the common dimensions of bacterial cells. Organoclays synthesized from smectite clay and the quaternary ammonium compound hexadecyltrimethylammonium (HDTMA) were also used to examine pesticide desorption rate and bioavailability. In these sorbents, the HDTMA is used to replace inorganic exchangeable cations of smectite clay. A sorptive phase is formed by agglomeration of the C-16 alkyl tails of HDTMA on the clay surfaces and in the interlayer regions. These sorbents are well defined compositionally, and can be produced synthetically to generate particles of known sizes. Bioavailability assays were conducted with these HDTMA-modified smectite clays of different particles sizes (< 0.25 to 1 mm in diameter) using *Alcaligenes* sp. strain NP-Alk, which cannot access soil-sorbed naphthalene directly (Crocker et al. 1995). For this organism, naphthalene sorbed to large HDTMA-clay aggregates remained unavailable for mineralization. As the clay aggregate size decreased, the rate and extent of desorption and mineralization increased progressing from large aggregates to small aggregates to unaggregated clay. The availability of sorbed naphthalene to strain NP-Alk was strictly dependent on the rate of desorption, which is inversely related to particle size (Crocker et al. 1995).

Guerin and Boyd (1997) evaluated bioavailability of naphthalene associated with various natural and synthetic sorbents. *Pseudomonas putida* strain 17484 had direct and immediate access to a portion of sorbed naphthalene by all natural sorbents and facilitated the desorption of additional naphthalene for degradation. Naphthalene sorbed by forest soils, however, was less available than naphthalene sorbed by agricultural soils or river sediments. This may be attributed to differences in the quality of the soil organic matter. Sorption to granular activated carbon virtually precluded naphthalene degradation, whereas naphthalene sorbed to XAD-2 resin, HDTMA-modified smectite, and Tenax was degraded by strain 17484. These results suggest that sorbent porosities and particle size, as well as strength of naphthalene sorption influence bioavailability.

4.3 Aging

Another important factor influencing the bioavailability of organic contaminants is the contact time between the contaminant and soil/sorbent, often referred to as aging. Aging often increases the sorption of organic chemicals by allowing more time for the chemicals to partition deeper into

the polymeric matrix of soil organic matter and to sorb into micro-voids or microporous minerals (Luthy et al. 1997). There is evidence that the bioavailability of organic chemicals decreases with time. Ethylene dibromide (EDB), a soil fumigant with relatively high water solubility, volatility and biodegradability, was reported to persist in field-weathered soils for up to 19 years after its last application (Steinberg et al. 1987). When ^{14}C-EDB was added to these soils in laboratory experiments, it was rapidly degraded by indigenous microbial populations, whereas field-aged EDB contained within the same sample was completely resistant to biodegradation. Scribner et al. (1992) compared the sorption/desorption behavior and bioavailability of field weathered (aged) simazine residues from a 20-year continuous cornfield to that of ^{14}C-simazine recently added to the same soil. The apparent sorption coefficients of the aged residues determined from 24 to 48 hour desorption experiments were 15 times higher than sorption coefficients of added simazine. Aged simazine residues were also shown to be biologically unavailable to sugar beet and to indigenous microbial populations whereas recently added simazine showed herbicidal damage to sugar beet and was substantially biodegraded in soil from the continuous corn field (Scribner et al. 1992). Guerin and Boyd (1993) evaluated how aging of naphthalene affected the bioavailability of soil-sorbed naphthalene to *Pseudomonas putida* strain ATCC 17484, which can access sorbed naphthalene. The results showed that aging of naphthalene in sterile soil slurries caused a significant decrease in the ability of strain 17484 to access the pool of sorbed naphthalene. The initial mineralization rates began diminishing with increasing naphthalene-soil contact time, and after one year they plateaued almost exactly at the rate predicted if all sorbed substrate was unavailable (Guerin and Boyd 1993). Feng et al. (2000) showed that the extent of bioavailability of soil-sorbed biphenyl decreased with increased aging. The decrease in availability was most pronounced at the early stage (< 80 days) of the aging period. The diminishing bioavailability of soil-aged chemicals has also been reported for phenanthrene and 4-nitrophenol (Hatzinger and Alexander 1995) and atrazine (Kelsey et al. 1997; Chung and Alexander 1998). Comparison of sorbent effects in an atrazine aging experiment illustrated the importance of soil organic matter. Bioavailability experiments using *Pseudomonas* sp. ADP showed that there was no dimunition of either atrazine mineralization rates or extents over a one-year aging period for K-saturated montmorillonite clay whereas for Colwood soil (7.8% OC) aging decreased the overall mineralization rates and extents progressively from two days to one year (Feng and Boyd unpublished data). Soil aging of organic contaminants can be viewed as a process in which initially surface sorbed chemical is slowly redistributed

to interior of the soil aggregate or organic matrix where it becomes less accessible or inaccessible to microorganisms.

4.4 Characteristics of Bacteria

Bioavailability is also influenced by certain, albeit poorly understood, characteristics of bacteria. To degrade soil-sorbed molecules, bacteria must either use sorbed molecule directly or facilitate desorption in some manner. Mechanisms underlying the apparent availability of sorbed chemicals are complex due to the divergent properties of chemicals considered, the resultant sorption/desorption mechanisms, the metabolic diversity of microorganisms, and the heterogeneity of soils. Several microbial-based mechanisms have been proposed for the access of soil-sorbed organic chemicals: (i) production of biosurfactants (Desai and Banat 1997; Alexander 1999); (ii) production of extracellular enzymes to degrade target compounds; (iii) microorganisms with high substrate affinity, which efficiently reduce concentrations of the substrate close to the cell surface (Bastiaens et al. 2000); (iv) reduction of the distance between cells and substrate by adhesion to sorbents (Alexander 1999; Bastiaens et al. 2000; Grosser et al. 2000); and (v) reduction of the distance between cells and substrate by means of motility and chemotaxis (Guerin and Boyd 1992).

Wu et al. (2003) evaluated bacterial characteristics related to some of the above-mentioned mechanisms using two biphenyl-degrading bacteria with different abilities to access soil-sorbed biphenyl. *Pseudomonas putida* strain P106 and *Rhodococcus erythropolis* strain NY05 had been shown to be able to access soil-sorbed biphenyl even when desorption was accounted for, and strain P106 had better accessibility to the pool of sorbed biphenyl (Feng et al. 2000). Wu et al. (2003) reported that both P106 and NY05 showed strong tendencies to attach to soils though NY05 was considerably more hydrophobic than P106. P106 was more motile and had a higher chemotactic response to biphenyl than NY05. No biosurfactant was detected in either culture. It appears that bacteria (P106 in this case) with higher chemotactic response and moderate cell surface hydrophobicity may access soil-sorbed biphenyl more efficiently. Park et al. (2003) also evaluated several characteristics of three atrazine-degrading bacteria that may affect degradation of soil-sorbed atrazine. Production of surfactants by three atrazine-degrading bacteria was not indicated by surface tension measurements. Access to sorbed atrazine seemed to be favored by chemotaxis and cell attachment to soils.

Wick et al. (2002) examined the physiological responses of anthracene-degrading *Mycobacterium* sp. LB501T to anthracene in batch

cultures when solid anthracene was supplied as a sole carbon source. This organism showed a high specific affinity for anthracene and grew as a confluent biofilm on solid anthracene. Cells grown on anthracene were significantly more hydrophobic and adhered better to Teflon and anthracene surfaces than those grown on glucose. No production of biosurfactants was observed. The results indicate that *Mycobacterium* sp. LB501T adapted to low substrate bioavailability by attachment and biofilm formation on the solid substrate.

5 Summary

Bioavailability of soil-sorbed organic chemical is a complex issue due to the simultaneous involvement of several processes including sorption/desorption, diffusion, and various chemical reactions and microbial transformations. The time dependency of bioavailability of chemicals, the physiological and metabolic diversity of microorganisms, and the spatial distribution of microorganisms capable of degrading target compounds in soils add complexity to this issue. Sorption often reduces the rate and extent of biodegradation and many sorbed substrates are not readily available; however, sorption does not necessarily prevent biodegradation from occurring. There is evidence that sorbed contaminants can be degraded by microorganisms or at least that desorption into bulk solution is not a prerequisite for biodegradation. Contaminant aging in soils generally reduces bioavailability of sorbed substrate. Future studies of biodegradation of sorbed chemicals should provide better mechanistic understanding and predictive models of bioavailability processes. Advance, however, is highly dependent on developing new and sensitive tools to measure physiochemical and microbiological parameters at microscale. The use of state-of-the-art biological tools, especially molecular techniques such as gene expression analysis and reporter systems represent perhaps the best opportunity to gain new mechanistic understanding of bioavailability. Another experimental challenge needs to be overcome is to obtain these parameters under conditions similar to the field soils. Improved understanding of bioavailability processes will guide realistic assessment of human health and ecological risks associated with sorbed pollutants, selection of appropriate remediation technologies, and determination of the cleanup goals.

Acknowledgement

This work was supported by the U.S. Department of Agriculture National Research Initiative Competitive Grants Program.

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11 Anticlastogenic and Antitoxic Actions Exerted by Humic Substances in Seedlings of Various Plants

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1 Introduction

Several natural and xenobiotic organic compounds, generally defined as environmental mutagens, possess the capacity of altering some genetic processes, such as mitotic division, which occur in meristematic plant cells (Grant 1994). In particular, some mutagens can behave as clastogens for their ability to produce breakage of chromosomes.

The antimutagenic action of a compound can be defined as the capacity to suppress or reduce mutagenic events, such as breakage and translocation of chromosomes and spindle disturbances, caused by a mutagen in an organism. The anticlastogenic activity is a particular aspect of the general antimutagenic action, and refers specifically to the reduction of chromosome breakages. Several tests are used to assess the (anti)clastogenicity

of various compounds in various macro- and microorganisms. These include the micronuclei (MN) test, the aberrant anatelophases (AAT) test, the comet assay, the Ames test, the C-mitotic effects, and the sister-chromatid exchange induction (Cozzi et al. 1993; Sujatha and Hegde 1998; Cotelle et al. 1999; Kalweit et al. 1999; Mueller et al. 1999; Vijayalaxmi and Venu 1999).

Humic acids (HA) and fulvic acids (FA) are the main components of humic substances (HS), which are the most chemically and biochemically active and widely spread fractions of nonliving natural organic matter in all terrestrial and aquatic environments. They comprise a chemically and physically heterogeneous group of substances with colloidal, polydispersed, polyelectrolyte characteristics and mixed aliphatic and aromatic nature (Senesi and Loffredo 1999).

Besides several other properties, HS are known to exert a number of direct and indirect biological effects on plants, including morphological, physiological and biochemical effects (Chen and Aviad 1990; Clapp et al. 2001; Varanini and Pinton 2001; Nardi et al. 2002) and genetic effects (Sato et al. 1986; Gichner et al. 1990; De Marco et al. 1995; Ferrara et al. 2001). In particular, HS of various origin and nature and at various concentrations can exert synergistic or antagonistic effects on the growth of plants treated with xenobiotic molecules (Senesi et al. 1990; Senesi and Loffredo 1994).

Relatively little information is available on the genetic actions that HS can exert in plants exposed to mutagens, although a mutagenic or an antimutagenic action has been observed in whole living organisms and single cells on dependence on the origin and nature of HS and the organism examined. In particular, the mutagenic activity of aquatic HAs and FAs has been investigated on bacteria and animal cells (Meier et al. 1983; Matsuda et al. 1991; Cozzi et al. 1993; Watt et al. 1996), and the antimutagenic behavior of HS has been also studied in several organisms (Sato et al. 1986, 1987; Gichner et al. 1989, 1990; Cozzi et al. 1993; De Marco et al. 1995, 1999; Ferrara et al. 2000, 2001).

The objective of this paper was to investigate the anticlastogenic and antitoxic effects exerted by HS of various origin and nature on several monocotyledon and dicotyledon herbaceous plant species treated with different mutagenic and phytotoxic compounds.

2 Materials and Methods

2.1 Humic Substances, Mutagenic Compounds and Plant Species Used

The HS samples used in this work were obtained from the Standard and Reference Collection of HAs and FAs of the International Humic Substances Society (IHSS), with the exception of the HA from an alluvial soil. The origin and nature of HS, with the corresponding codes, abbreviations and concentrations used, are shown in Table 1.

The mutagenic compounds used with the corresponding abbreviations and concentrations used, are listed in Table 2.

Eleven plant species (Table 3) were preliminarly tested for their response to the Feulgen staining method (described below), which is essential for the efficient microscope observation of genetic anomalies of cells. This, in order to select plants to be successively used in the experiments with the mutagenic compounds.

2.2 Preliminary Experiment

A defined number of seeds of each plant was germinated in Petri dishes kept in a Phytotron growth chamber at 21 ± 1°C in the dark. Root tips (~ 2 mm) were collected after 5 or 7 days of germination on dependence on the plant species, and subjected to Feulgen staining procedure before the preparation of permanent slides for the observation at an Olympus CX40 microscope.

In brief, the Feulgen staining procedure consists in: (a) fixation of root tips in Carnoy's solution I (ethanol and acetic acid, 3:1 v/v); (b) staining with Schiff's reagent; and (c) two successive immersions in 95% ethanol and histolemon Erba baths. More details on the procedure can be found in Ferrara et al. (2001).

Only four plant species, i.e., *Vicia faba*, *Allium cepa*, *Pisum sativum* and *Triticum turgidum*, responded adequately to the Feulgen procedure, and were considered for use in the successive experiments. These species, together with the corresponding mutagenic compounds used and the mutagenicity tests adopted (see below in Sect. 2.4.) are listed in Table 4.

Table 1. Origin and nature of humic substances used with corresponding codes, abbreviations and concentrations

Origin and nature of humic substances	IHSS Code	Abbreviation	Concentrations used (mg L^{-1})
Humic acids			
Elliott soil (Mollisol)	1S102H	MHA	20, 50, 200, 500
Summit hill Soil	1R106H	SHHA	20, 200
Pahokee peat	1R103H	PHA	20, 50, 200, 500
Leonardite	1S104H	LHA	20, 50, 200, 500
Nordic lake	1R105H	NHA	50, 500
Alluvial soil		ASHA	10, 100
Fulvic acids			
Elliott soil (Mollisol)	1S102F	MFA	20, 50, 200, 500
Pahokee peat	1R103F	PFA	20, 50, 200, 500
Nordic lake	1R105F	NFA	50, 500
Suwannee river	1R101F	SRFA	50, 500

Table 2. Mutagenic compounds used with corresponding abbreviations and concentrations

Mutagenic compound	Abbreviation	Concentration (mg L^{-1})
Maleic hydrazide	MH	2.5, 5.0, 7.5, 10
Colchicine	COL	1, 10, 100
Alachlor	ALA	1, 10
2,4-D	2,4-D	0.01, 0.1, 1
Glyphosate	GLY	10, 100, 1000

Table 3. Plant species tested

Common name	Plant species	Botanical group
Broad bean	*Vicia Faba*	[a]D
Onion	*Allium cepa*	[a]M
Pea	*Pisum sativum*	D
Durum wheat	*Triticum turgidum*	M
Bean	*Phaseolus vulgaris*	D
Rape	*Brassica napus*	D
White mustard	*Sinapsis alba*	D
Flax	*Linum usitatissimum*	D
Tomato	*Lycopersicon escuentum.*	D
Melon	*Cucumis melo*	D
Sunflower	*Heliantus annuus*	D

[a] M: monocotyledon; D: dicotyledon

Table 4. Plant species positive to the Feulgen method, mutagenicity coumpounds used and mutagenic tests applied

Plant species	Mutagenic compound	Mutagenicity test
Vicia faba	MH	MN, AAT
	COL	HC, PC
	2,4-D	MN, AAT
	GLY	MN, AAT
Allium cepa	MH	MN, AAT
	COL	HC, PC
	2,4-D	MN, AAT
	GLY	MN, AAT
Pisum sativum	MH	MN, AT
Triticum turgidum	MH	MN, AT
	ALA	MN, AT

2.3 Main Experiment

In the main experiment, germination was achieved by treating six to twenty seeds of each of the four selected plant species placed in Petri dishes, in a Phytotron growth chamber at 21 ± 1°C in the dark (see above), with 12 mL (*V. faba*) or 8 mL (*A. cepa, P. sativum, T. turgidum*) of the following test solutions: (a) distilled H_2O (positive control); (b) each mutagen alone (negative control); (c) each HA or FA alone; and (d) each HA or FA in combination with each mutagenic compound. The concentrations of the mutagens and HAs and FAs used in the experiments are indicated in Tables 1 and 2. The mixtures HA or FA and mutagen were mechanically shaken for 24 h at room temperature (20 ± 1°C) before addition to the seeds. The pH value of all solutions used ranged from 6 to 7. All experiments were triplicated.

As in the preliminary experiment, root tips were collected after 5–7 days, subjected to the Feulgen staining procedure as described above, then prepared adequately as permanent slides, and finally subjected to microscope observation. For each treatment, fifteen root tips (5 × 3 replicates) were prepared and 30,000 cells (2,000 cells per root tip) were examined in the case of MH, and 150 metaphases (5 × 3 replicates) were examined in the case of COL.

2.4 Mutagenicity and Toxicity Tests

The mutagenicity level was estimated by two different assays on dependence on the mutagen tested: (a) counting of frequencies of micronuclei (MN), aberrant anatelophases (AAT) and regular anatelophases (RAT) in the case of MH (Ferrara et al. 2000, 2001); and (b) counting of polyploid cells (PC) and hyperdiploid cells (HC) in the case of COL (Sbrana et al. 1993). In particular, the MN and AAT tests consist in identifying and counting the MN and AAT present in the treated cells. The MN are portions of extranuclear DNA with a diameter no larger than 1/3 of the main nucleus, and consist of chromosome fragments originated from a clastogenic event or complete chromosomes that do not replicate or segregate correctly because of spindle anomalies. The AAT consist of abnormal cell divisions showing chromosomal bridges, isolated DNA fragments and/or lagging and sticking chromosomes.

Some HS samples were also tested, either alone or in combination with some mutagenic compounds, for their possible antitoxic effect on the seedlings of some plant species. In particular: (a) the sample ASHA at a concentration of 10 or 100 mg L^{-1}, alone or in combination with 1 or 10 mg L^{-1} of ALA, was tested on *T. turgidum*; and (b) samples SHHA, PHA, PFA, LHA, SHA and SFA at concentrations of 20 or 200 mg L^{-1}, alone or in combination with 10 mg L^{-1} MH, were tested on *V. faba*. The antitoxic effect was evaluated by measuring some biometrical parameters such as

length and dry weight of shoots and roots. In the case of *V. faba*, biometrical parameters were measured on 5-day grown seedlings before cutting root tips for the antimutagenic observations. For *T. turgidum*, the measurements were made on germinated seedlings grown for 14 days in glass pots in the presence of the same test solutions used for seed germination.

2.5 Statistical Analysis of Data

Experimental data obtained were statistically analyzed by one-way analysis of variance (ANOVA) at both 95% and 99% confidence levels. The mean values were separated by using the least significant difference (LSD) test in all cases, except in the experiments with MH alone where the averages were separated by using the Duncan's test. For both antimutagenic and antitoxic evaluations, the mean values measured in the HS-alone treatments were statistically compared to those of the positive control (H_2O), whereas the mean values obtained in the experiments using the combinations HS + mutagen were compared to those of the negative control (MH, COL or ALA).

3 Results and Discussions

3.1 Anticlastogenic Action

Results of preliminary experiments showed that only root tip cells of *V. faba*, *A. cepa*, *P. sativum* and *T. turgidum* responded positively to the Feulgen procedure (Table 4), with an evident appearance of MN and ATT anomalies (Fig. 1), which were more abundant in *V. faba* than in *P. sativum* (Fig. 2). The other plant species examined yielded a poor staining of the nuclear material, thus discouraging their use in successive experiments.

Among the mutagens tested on each plant species (Table 4), those that produced evident clastogenic alterations were: (a) MH on *V. faba*, *A. cepa*, *P. sativum*, and *T. turgidum*; (b) COL on *V. faba* and *A. cepa*; and (c) ALA on *T. turgidum*. The effects measured for MH and ALA on *T. turgidum*, and for 2,4-D and GLY on *V. faba* and *A. cepa*, were not statistically significant, thus the related data will not be considered further in this section.

In general, with the exception of AAT on *P. sativum*, treatments of any plant species with each HS sample alone did not determine MN and AAT frequencies statistically different from the positive control (Table 5 and Fig. 3). These results suggested the absence of a significant clastogenic activity exerted by HS alone (Sato et al. 1986; De Marco et al. 1995, 1999).

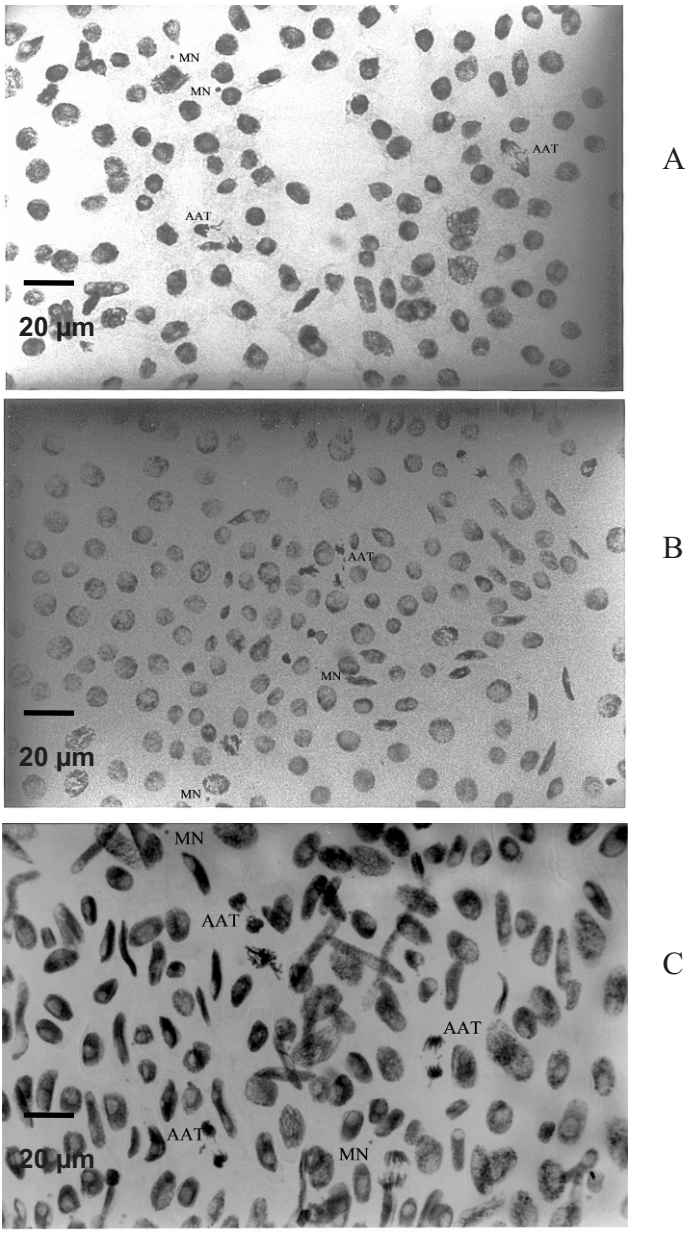

Fig. 1. Root tip cells of *Vicia faba* (**A**), *P. sativum* (**B**) and *A. cepa* (**C**) treated with 10 mg/L of maleic hydrazide (MH) showing the presence of micronuclei (MN) and aberrant anatelophases (AAT). Magnification 400x.

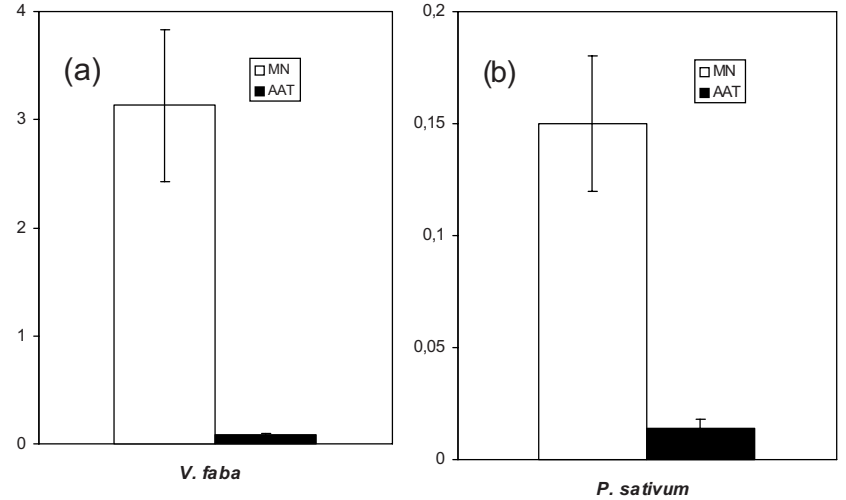

Fig. 2. Frequency (%) of MN and AAT in 30,000 root-tip cells of (**a**) *V. faba* and (**b**) *P. sativum* expressed as an average of all treatments including the positive and negative controls, HS treatments and HS + MH treatments. The vertical line on each bar indicates the standard error (n = 3).

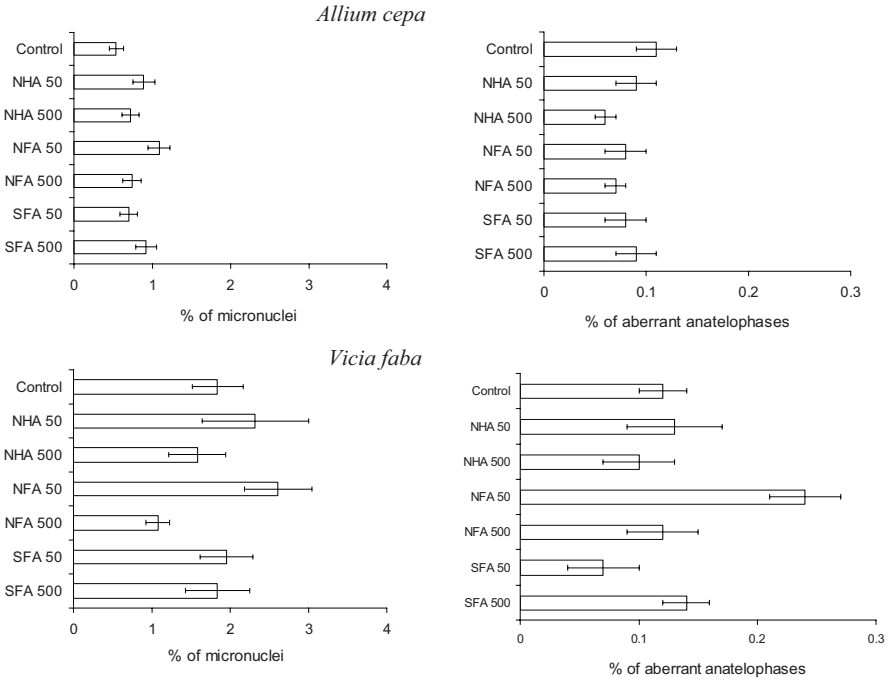

Fig. 3. Frequency (% on 30,000 cells for each treatment) of MN and AAT in *A. cepa* and *V. faba* root tip cells. Each treatment results not significantly different with respect to the control according to the LSD test.

Table 5. Frequency (%) on 30,000 cells for each treatment of micronuclei (MN) and aberrant anatelophases (AAT) in *Vicia faba* and *Pisum sativum* root tip cells treated with HA or FA alone

Treatment	*Vicia faba*		*Pisum sativum*	
	MN	AAT	MN	AAT
H_2O (positive control)	0.86 ± 0.22	0.05 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
SHHA, 20 mg L^{-1}	2.04 ± 0.30 ns	0.11 ± 0.02 ns	0.05 ± 0.01 ns	0.00 ± 0.00 **
SHHA, 200 mg L^{-1}	1.66 ± 0.14 ns	0.08 ± 0.02 ns	0.02 ± 0.01 ns	0.01 ± 0.01 *
PHA, 20 mg L^{-1}	1.11 ± 0.38 ns	0.04 ± 0.02 ns	0.04 ± 0.02 ns	0.03 ± 0.01 ns
PHA, 200 mg L^{-1}	0.87 ± 0.19 ns	0.06 ± 0.02 ns	0.06 ± 0.01 ns	0.01 ± 0.01 *
PFA, 20 mg L^{-1}	0.80 ± 0.14 ns	0.08 ± 0.02 ns	0.07 ± 0.03 ns	0.01 ± 0.01 *
PFA, 200 mg L^{-1}	0.85 ± 0.27 ns	0.04 ± 0.02 ns	0.04 ± 0.01 ns	0.00 ± 0.00 **
				SD (0.05P): 0.02 SD (0.01P): 0.03

The symbols **, *, and ns refer, respectively, to a difference significant at 0.01P, a difference significant at 0.05P, and a nonsignificant difference, according to the LSD test.

Differently, and in agreement with previous findings (De Marco et al. 1995, 1999), both MN and AAT were observed in all treatments with MH (Fig. 1), including the positive control (H_2O treatment). However, with respect to the positive control, the treatment with MH alone at different concentrations produced a significant increase of the clastogenic effect in *V. faba*, *A. cepa* and *P. sativum* (Ferrara et al. 2004) (Fig. 4). The MN and AAT frequencies increased as a function of MH concentration, but the differences were significant at the Duncan's test at $P<0.05$ only for AAT (Fig. 4). With respect to the positive control, the increases of frequencies measured for *V. faba*, *A. cepa* and *P. sativum* were for MN, respectively up to 9.8, 10 and 14 times, and for AAT, respectively up to 7.6, 2.8 and 1.8 times.

All the combinations HS + MH reduced extensively the genetic anomalies caused by MH, thus indicating that HS exerted an evident anticlastogenic activity in the three species, whose extent was a function of HS source, nature and concentration. In particular, the effects of the combinations HS + MH on the relative frequencies (%) of MN and AAT in root tip cells of *V. faba*, *P. sativum* and *A. cepa*, are shown in Figs. 5, 6 and 7, respectively, as referred to the frequency in the negative control (MH) assumed 100%. In general, the MN test resulted more efficient than the AAT test in measuring clastogenic/anticlastogenic effects. Further, *V. faba* responded better that the other species to the tests possibly because of larger

chromosome size (Kanaya et al. 1994) and/or greater amount of DNA per nucleus (26.7 pg in *V. faba* and 9.8 pg in *P. sativum*) and/or greater sensitivity to clastogenic tests (Grant and Owens 2001).

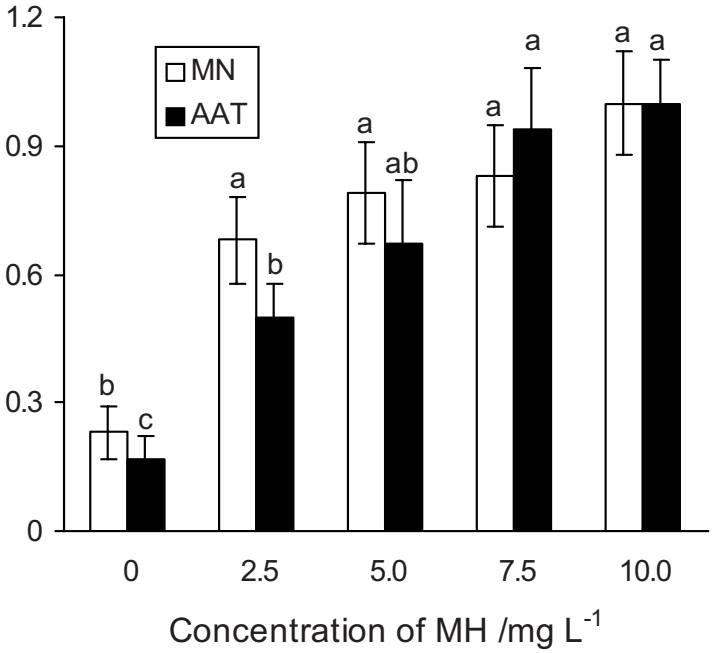

Fig. 4. Relative frequency of MN and AAT in 30,000 root-tip cells of *V. faba* treated with MH at different concentrations, with respect to the frequency measured for the treatment with 10 mg L^{-1} of MH.

In particular, with respect to the MH-alone treatment, the reduction of both anomalies in *V. faba* resulted highly significant for almost all the combinations (Fig. 5). The greatest reduction of MN frequency was measured for the combinations of NFA at 50 mg L^{-1} + MH (77.6%) and PHA and PFA at 20 mg L^{-1} + MH (74.5 and 68.5%, respectively), and of AAT frequency for the combinations NHA at 500 mg L^{-1} + MH and NFA at 50 mg L^{-1} + MH (75% for both combinations).

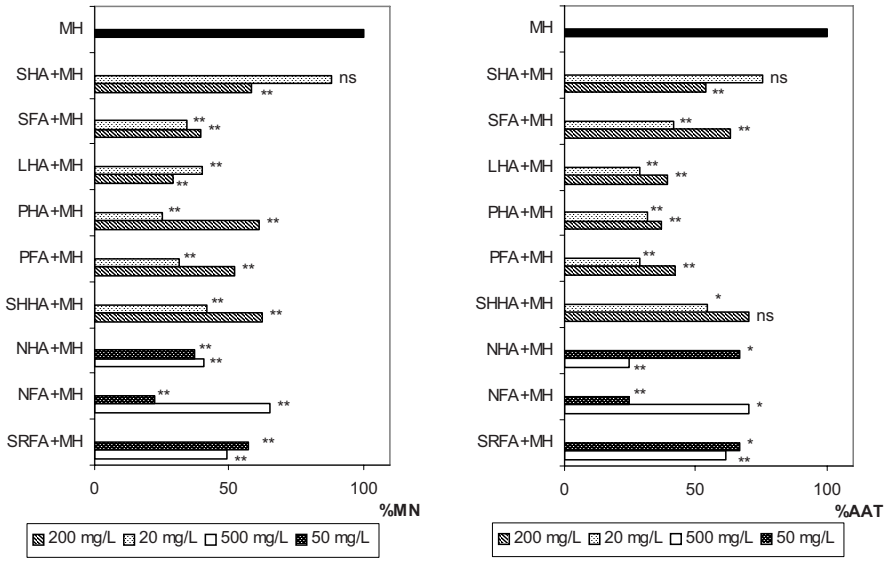

Fig. 5. Effect of the combinations of MH with each HS at various concentrations on the relative frequency (%) of MN (*left*) and AAT (*right*) in *V. faba* root tip cells referred to the control (MH alone, frequency 100%). The symbols **, *, and ns refer, respectively, to a difference significant at 0.01P, a difference significant at 0.05P, and no significant difference according to the LSD test.

In the experiments with *P. sativum* the greatest reductions of MN, and especially AAT, were generally obtained at high HS concentration, whereas at low HS concentration the greatest reductions were obtained for the combinations PHA + MH and PFA + MH (Fig. 6). In the case of *A. cepa*, the various HS tested at high concentration (500 mg L^{-1}) behaved almost similarly in reducing the frequency of either MN or AAT, and were more efficient in reducing AAT frequency than MN frequency (Fig. 7).

As expected, a lower number of RAT, that is, a mitodepressive effect, is observed in plant species treated with MH alone (data not shown) (Ferrara et al. 2001). With respect to the treatment with MH, any combination HS + MH appeared not to modify the mitotic activity (RAT number) of cells in *V. faba*, whereas it caused a slight reduction of the RAT number in *P. sativum*, and yielded contrasting results in *A. cepa* (data not shown) (Ferrara et al. 2001).

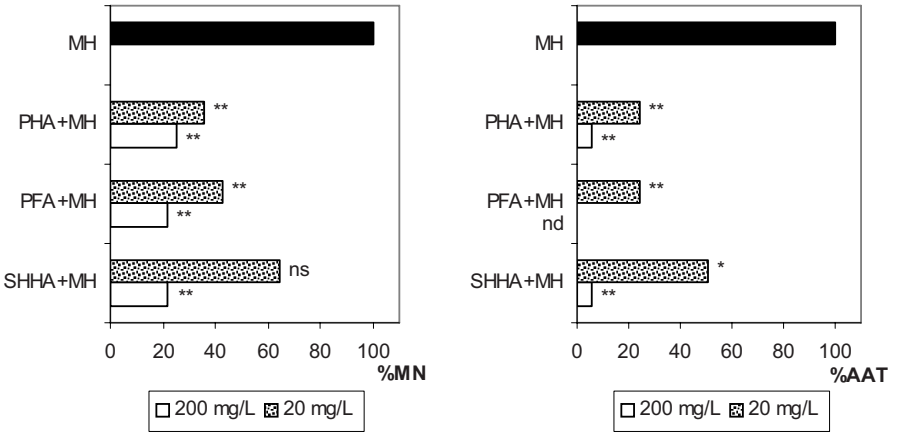

Fig. 6. Effect of the combinations of MH with each HS at 20 or 200 mg L^{-1} on the relative frequency (%) of MN (*left*) and AAT (*right*) in *P. sativum* root tip cells referred to the control (MH alone, frequency 100%). The symbols **, *, and ns refer, respectively, to a difference significant at 0.01P, a difference significant at 0.05P, and no significant difference according to the LSD test.

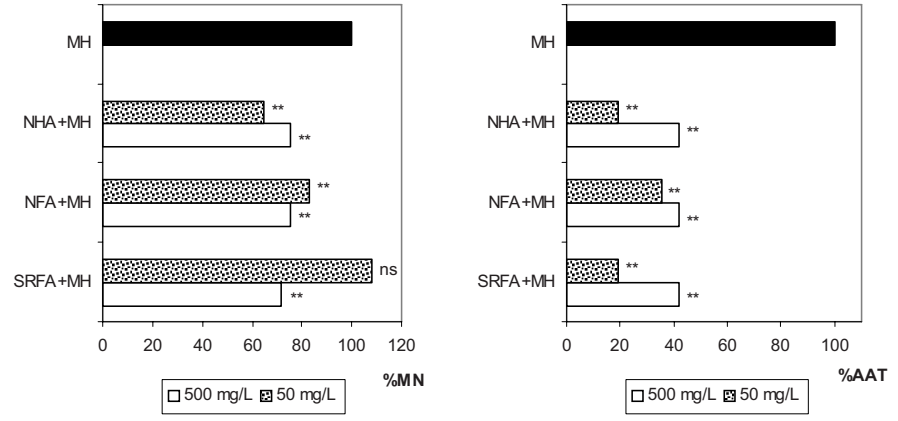

Fig. 7. Effect of the combinations of MH with each HS at 50 or 500 mg L^{-1} on the relative frequency (%) of MN (*left*) and AAT (*right*) in *A. cepa* root tip cells referred to the control (MH alone, frequency 100%). The symbols ** and ns refer, respectively, to a difference significant at 0.01P and no significant difference according to the LSD test.

Only *A. cepa* and *V. faba* showed the presence of PC and HC in COL-treated root tip cells (negative control) (Fig. 8), whereas cells grown in the positive control (H_2O) did not show any PC (data not shown). With respect to the COL-alone treatment, the combinations HS + COL apparently produced anticlastogenic effects less pronounced than those of HS + MH combinations described above. In particular, only the combinations of COL with LHA, PHA and PFA at lower concentration produced a statistically significant reduction of PC frequencies in cells of *A. cepa* (Fig. 9). Further, the number of HC in root tip cells of *A. cepa* and *V. faba* treated with COL (negative control) was much higher than that in the positive control, and no significant reduction of HC frequencies was observed for any combination HS + COL (data not shown).

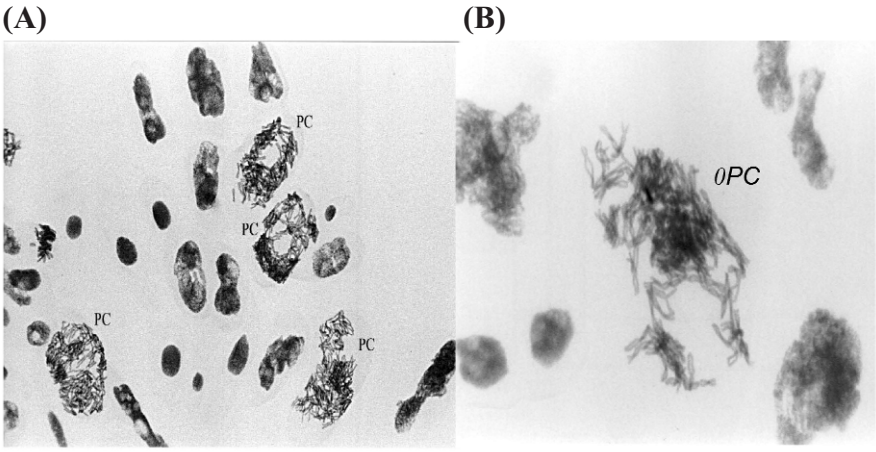

Fig. 8. Root tip cells of *A. cepa* treated with 100 mg L^{-1} COL showing the presence of polyploid cells (PC) at magnification of 400× (**A**) and 1000× (**B**).

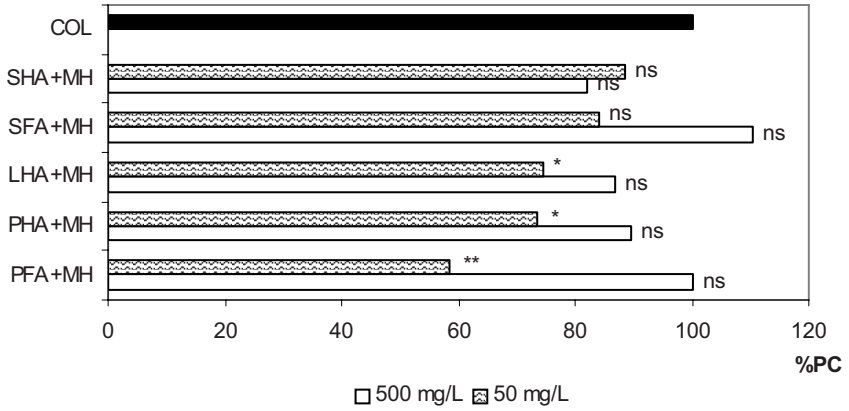

Fig. 9. Effect of the combinations of COL with each HS at 50 or 500 mg L^{-1} on the relative frequency (%) of PC in *A. cepa* root tip cells referred to the control (COL alone, frequency 100%). The symbols **, *, and ns refer, respectively, to a difference significant at 0.01P, a difference significant at 0.05P, and nonsignificant difference, according to LSD test.

3.2 Antitoxic Action

In Fig. 10 the effects are shown of samples SHHA, PFA, PHA, LHA, SFA and SHA at concentrations of 20 and 200 mg L^{-1}, either alone or in combination with 10 mg L^{-1} of MH, on the length and dry weight of the primary root of 5-days grown seedlings of *V. faba.* With respect to the positive control (C), only samples PFA, PHA and LHA used alone at both concentrations were able to increase significantly the primary-root elongation of *V. faba* seedlings, whereas the effect on primary-root dry weight was generally much smaller. The maximum increase of root length and dry weight (~150% and 100% of the control treatment, respectively) was observed in the treatment with PFA at 20 mg L^{-1}. These results confirm previous findings by other authors about stimulation of primary-root growth by HS of different origin and nature (Chen and Aviad 1990; Nardi et al. 2002). Small, nonsignificant variations of primary-root length and dry weight were observed with the other HS samples.

Besides clastogenic effects, MH also produced phytotoxic effects on *V. faba* seedlings by reducing root length and dry weight, respectively to 70 and 62%, with respect to the positive control (H_2O) treatment. However, the combinations of MH with PFA, PHA and LHA at either concentration, not only suppressed the toxic effect of MH, but also stimulated

primary-root growth (Fig. 10). The largest effect was measured for the combination LHA + MH, with about 140 and 100% increase of, respectively, primary-root length and dry weight, with respect to the MH-alone treatment.

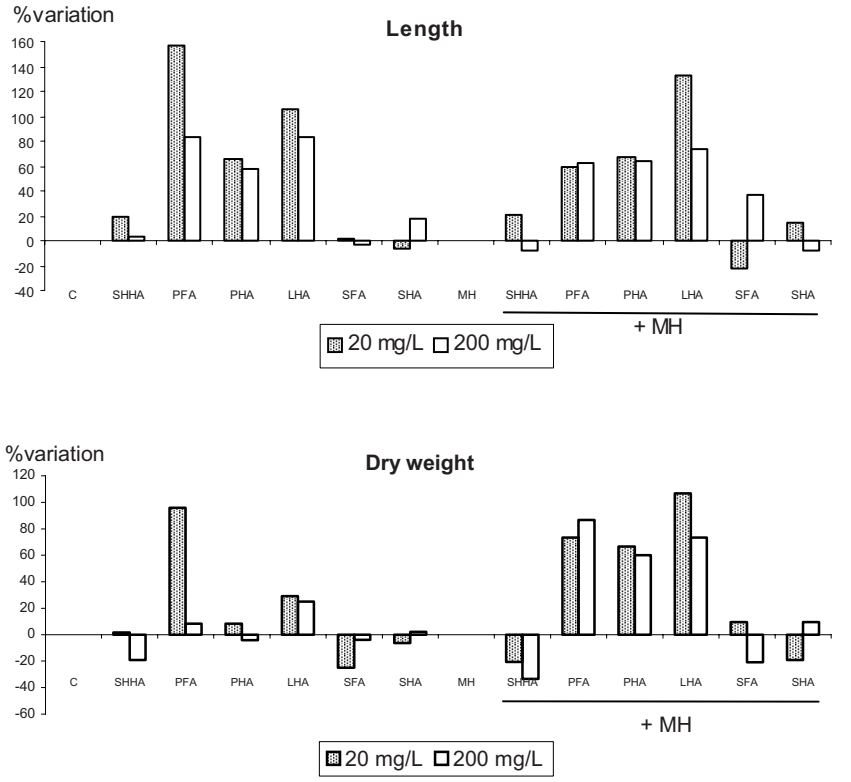

Fig. 10. Effects of different HS samples at 20 and 200 mg L^{-1}, used either alone or in combination with MH at 10 mg L^{-1}, on the length and dry weight of primary root of *V. faba* seedlings. Data of HS and HS + MH treatments are expressed as the percentage of the variation observed with respect to the H_2O treatment (C) and to the MH treatment, respectively.

In Fig. 11 the effects are shown of ASHA at concentrations of 10 or 100 mg L^{-1}, alone and in combination with ALA at concentrations of 1 and 10 mg L^{-1}, on the length and dry weight of primary root and shoot of 14-days grown seedlings of *T. turgidum*. The presence in the growth medium of ASHA alone at both concentrations produced a significant increase of primary-root and shoot lengths and shoot dry weight, with respect to the positive control (H_2O). The greatest effect, with respect to the

control, was exhibited on shoot length, with increases of 70% (at low concentration) and 80% (at high concentration), and shoot dry weight.

The herbicide ALA at both concentrations depressed markedly seedling growth of *T. turgidum* by reducing root and shoot length and weight, and produced evident phytotoxic symptoms, such as leaf chlorosis and altered root morphology. In particular, with respect to the control treatment (H_2O), ALA at 1 and 10 mg L^{-1} reduced root length, respectively to 88 and 17%, shoot length to 72 and 40%, root dry weight to 83 and 18%, and shoot dry weight to 86 and 46%.

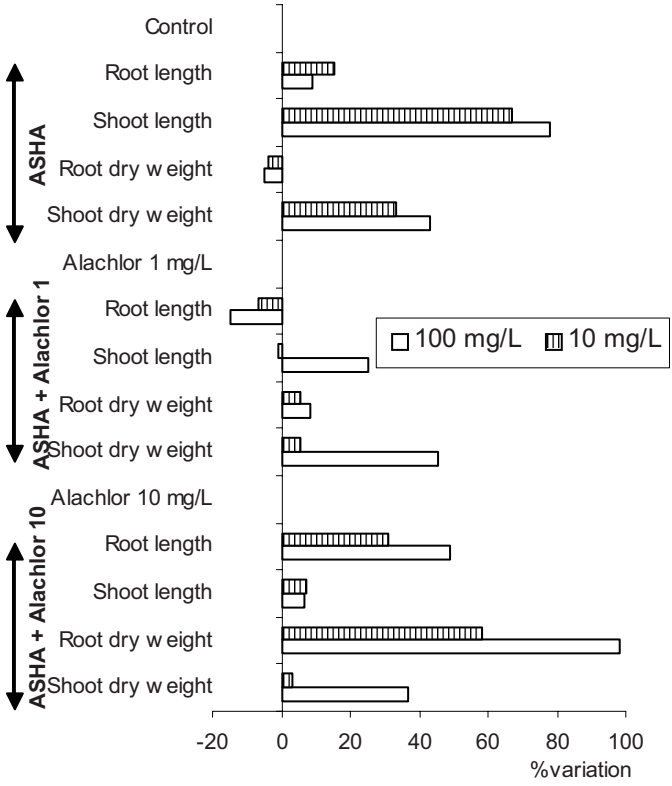

Fig. 11. Effects of ASHA at 10 or 100 mg L^{-1}, alone and in combination with 1 or 10 mg L^{-1} ALA, on the length and dry weigth of primary root and shoot of *T. turgidum* seedlings. Data of ASHA and ASHA + ALA treatments are expressed as the percentage of the variation observed with respect to the control (H_2O treatment) and to the ALA treatments, respectively.

The presence of ASHA in the growth medium, especially at higher concentration, apparently attenuated the phytoxicity induced by ALA on *T. turgidum* seedlings (Fig. 11). In particular, with respect to the ALA alone treatment, the combination of ALA at 1 mg L^{-1} with ASHA at 100 mg L^{-1} produced a significantly enhanced growth of shoots and a slightly reduced or increased primary-root elongation and dry weight, respectively. An opposite effect was measured in the treatments ASHA + ALA at 10 mg L^{-1}, in which primary-root length and dry weight increased very significantly, and shoot length and dry weight only slightly, with respect to the ALA alone treatment. The effects were generally more pronounced at high concentration of ASHA. In a previous study on tomato seedlings treated with ALA and other herbicides, a marked attenuation of the toxic symptoms was found when soil HAs were added to the herbicide in the growth medium (Senesi et al. 1990).

4 Summary and Conclusions

Several plant species were investigated preliminarly for their response to a number of clastogenic and phytotoxic compounds, but only four of them, *V. faba*, *A. cepa*, *P. sativum* and *T. turgidum*, responded positively to the study of genetic anomalies and toxicity exerted by the mutagens MH and COL, and measured as MN and AAT frequencies. When tested alone, a number of HS samples of different origin and nature showed no statistically different variations of MN and AAT frequencies with respect to those of the positive control (H_2O), thus HS were assumed not to exert by themselves any significant clastogenic activity on the four plants investigated.

More important, the HS samples studied appeared to exert an anticlastogenic action (i.e., a decrease of MN and AAT frequencies with respect to MH or COL alone) in plant seedlings studied, at an extent that varied as a function of their origin, nature and concentration, and the plant species and the mutagen used. The highest anticlastogenic effect was obtained for HS of aquatic, peat and leonardite sources in *V. faba* and *A. cepa* treated with the mutagen MH. In general, HAs and FAs exhibited a similar anticlastogenic behavior.

Besides an anticlastogenic activity, some HS appear to possess also an antitoxic activity, i.e., they were able not only to suppress plant growth depression caused by MH and ALA, but also stimulate growth. Peat, leonardite and alluvial soil HS yielded the best results also for the antitoxic activity.

Attempts made to possibly relate the extent of anticlastogenic and/or antitoxic activities of HS studied to their main compositional, structural, chemical and physico-chemical properties, including carboxyl and phenolic group contents, aromaticity, aliphaticity, organic free radical concentration, and others, have been mostly unsuccessful. However, the greater bioactivity of PHA and PFA, with respect to other HS, can tentatively be attributed to their greater carboxyl group content and aromaticity (Ferrara et al. 2004). In conclusion, the mechanism(s) of action of HS as anticlastogens and antitoxic is far to be understood in detail, although it can be hypothesized that the mutagen molecules can be adsorbed and/or somehow inactivated by interaction with some reactive groups of HS, thus resulting in a decreased availability for root adsorption.

Because of these important properties, a future application of HS can be expected as protecting agents for the prevention or at least limitation of genetic damages that may be caused by genotoxic environmental pollutants in plants, and also in microorganisms, animals, and even humans.

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12 Soil Heavy Metal Pollution and Microbial Communities: Interactions and Response Assessment

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1 Heavy Metal Pollution

Heavy metal pollution of soils is one of the most serious problems of present day agriculture which negatively affects both crop yields and quality. Heavy metal pollution results by the disposal of concentrated metal wastes. First observations of the effects of heavy metals on soil microbial processes were reported by Lipman and Burgess in 1914. But only when the large adverse effects of emissions of heavy metals from smelters on surrounding ecosystems were observed in the 1960–70s, then it was realized that how severely soil microorganisms and soil microbial processes can become disrupted by elevated metal concentrations. Extreme metal contamination in the vicinity of smelters caused clearly visible effects such as accumulation of deep layers of organic matter on the soil surface through

inhibition of the activity of soil microorganisms and soil fauna (Freedman and Hutchinson 1980). When measures to limit the metal loading rates of soils due to the use of sewage sludge in agriculture were first introduced in many European countries during the 1970s, these limits were focused on protecting against negative effects on crop plants, on animals grazing on land to which sewage sludge had been applied and to protect man from metal exposure through the food chain. It was not until 20 years later that the effects of elevated heavy metal concentrations on soil microorganisms were taken into consideration in the drafting of legislation to regulate the agricultural use of sewage sludge (Witter 1992). EU mandatory limits were also established to prevent the build up of metal concentrations in agricultural soils. Several heavy metals are presently emitted in great quantities as a result of human activities (Table 1).

2 Soil Microbial Communities

Microbial communities in soil are extremely diverse, with estimates of as many as 13,000 species of bacteria present in per gram of soil (Torsvic et al. 1994). Although diversity has been assumed to confer greater stability on ecological systems, experimental evidence to demonstrate such a link is scarce. Microbial biomass which represents the living component of the organic matter of soil usually makes up less than 5% of soil organic matter (Dalal 1998), but it carries out many critical functions in the soil ecosystem. Microbial biomass is both a source and sink for nutrients in the soil. It participates in the C, N, P and S transformations, and plays an active role in the degradation of xenobiotic organic compounds. It also helps in the mobilization and immobilization of heavy metals and participates in the formation of soil structure, etc. (Nannipieri et al. 2002). As soil microorganisms play a vital role in maintaining soil productivity, thus, any thing that disrupts these microorganisms and their functions in soil could be expected to affect the long term soil productivity and sustainability and even the ecosystem stability.

Table 1. Concentration of total (T) and available (A) Cu, Zn and Cd in soils sampled at various distances from the copper and zinc smelter in China (from Li et al. 2006)

Sample no	Distance (km)	T-Cu (mg kg^{-1})	A-Cu (mg kg^{-1})	T-Zn (mg kg^{-1})	A-Zn (mg kg^{-1})	T-Cd (mg kg^{-1})	A-Cd (mg kg^{-1})
1	0.01	4895	1340	1133	392	28.8	7.3
2	0.20	1084	434	1037	186	34.0	7.4
3	0.60	751	366	881	106	24.5	7.1
4	0.80	473	261	848	121	22.2	5.8
5	1.00	464	254	691	107	22.4	4.1
6	1.20	416	95	717	138	20.4	2.5
7	5.00	46	6	96	8	6.9	Trace

3 Mechanisms of Heavy Metal Toxicity

Heavy metals are toxic because of their ionic properties. They bind to many cellular ligands and displace native essential metals from their normal binding sites (Wittekind et al. 1996). For example, arsenate can replace phosphate in the cell. Metals also disrupt protein by binding to sulfhydryl groups and nucleic acids by binding to phosphate or hydroxyl groups. As a result, protein and DNA conformation are changed and their function is disrupted (Bruce et al. 2003). For example, cadmium competes with cellular zinc and nonspecifically binds to DNA, inducing single-strand breaks (Alloway 1995). Metals may also affect oxidative phosphorylation and membrane permeability, as seen with vanadate and mercury (Muller et al. 2001). Microorganisms generally use specific transport pathways to bring essential metals across the cell membrane into the cytoplasm. Toxic metals can also cross membranes via diffusion or via pathways designed for other metals (Konopka et al. 1999). For instance, Cd^{2+} transport occurs via the

Mn^{2+} active transport system in *Staphylococcus aureus*. These metal-microbe interactions result in decrease microbial growth, abnormal morphological changes, and inhibition of biochemical processes in individual (Akmal et al. 2005a,b). The toxic effects of metals can be seen on a community level as well. In response to metal toxicity, overall community numbers and diversity decrease. Soil is a living system where all biochemical activities proceed through enzymatic processes. Heavy metals have also adverse effects on enzyme activities (Fig. 1).

4 Heavy Metal-Microbe Interactions and Microbial Response Assessment

The associated heavy metal can affect the bioavailability of the metal in question by additive, synergistic, or antagonistic effects. These interactions can be positive, negative, or nonexistent (Table 2). However, the gross microbial biomass and activity measurements seldom indicated whether the observed effects were due to changes in species composition or to reduced physiological capacities of the microbial community (Frostegard et al. 1996; Knight et al. 1997). Studies, using the plate count techniques, have demonstrated a shift in the composition of fungal species towards a more metal-tolerant community in the metal-contaminated soils (Yamamoto et al. 1985; Ueda et al. 1988). Usually a decrease in the commonly isolated genera as *Penicillium oidiodendron* and *Mortierella* spp. were observed in the metal polluted soils by Nordgren et al. (1983). Others, such as *Geomyces* and *Paecilomyces*, increased in abundance towards the metal source. *Penicillium* spp. were mostly dominant in soils polluted by copper mine drainage (Yamamoto et al. 1985).

Like fungi, soil bacteria also vary in their sensitivity to the metal pollution. There have been reports of effects on the bacterial community composition, generally showing an increase in gram-negative bacteria in metal contaminated soils (Zelles et al. 1994). An exception to this has been reported by Ross et al. (1981), who observed that Gram- negative bacteria were slightly more sensitive to Cr than Gram- positive ones. Since bacteria were seldom identified up to species level, conclusions on the effects of metals on bacterial species are hard to be drawn (Frostegard et al. 1996). Generally, the degree of tolerance of microorganisms to metal pollutants

varies in the order: fungi > bacteria > actinomycetes (Frostegard et al. 1993). A decrease in bacterial number within 24 hour of incubation in a Zn-spiked soil was observed by Ohya et al. (1985). In contrast, Frostegard et al. (1996) reported an increase in the overall fungal populations in the Cr or Zn contaminated soil. Therefore, metal pollution of soils often results in an increase in the fungal to bacterial ratio in soils (Hattori 1992). Genetic diversity is always present within species and may be crucial in determining the response of a population to changing conditions (Young 1994). Highly stable, uniform environments with abundant resources allow the dominance of particularly competitive species, whereas moderate stresses may decrease the likelihood of competitive exclusion.

Fewer studies have attempted to examine more subtle effects of heavy metal pollution on the structure of microbial communities or on the genetic diversity of particular groups of organisms. Most of the studies used a physiological approach in which the ability of the bacterial microbial community to utilize a variety of substrates was tested to compare the relative activities of different groups of microorganisms and this ability has been related to metal tolerance (Reber 1992; Doelman et al. 1994). These studies have served to highlight the subtle effects of heavy metals on the soil microbial community (Fig. 2). Evidence from the field experiments suggests that under long-term metal stress a change in the genetic structure of the soil microbial community is produced (Amann et al. 1996). A decrease in the total soil microbial biomass under chronic metal stress has been observed in many field experiments, but is likely to be preceded by changes in community structure (Kozdroj and van Elsas 2000). A decreased size of the microbial biomass could at least partially be explained by physiological causes such as a decrease in the microbial substrate utilization efficiency (Fig. 3) and an increased maintenance energy requirement under heavy metal pollution. A decrease in the number of substrates which can be utilized and thus a reduction in the efficient exploitation of all ecological niches may also explain the decrease in the size of the biomass.

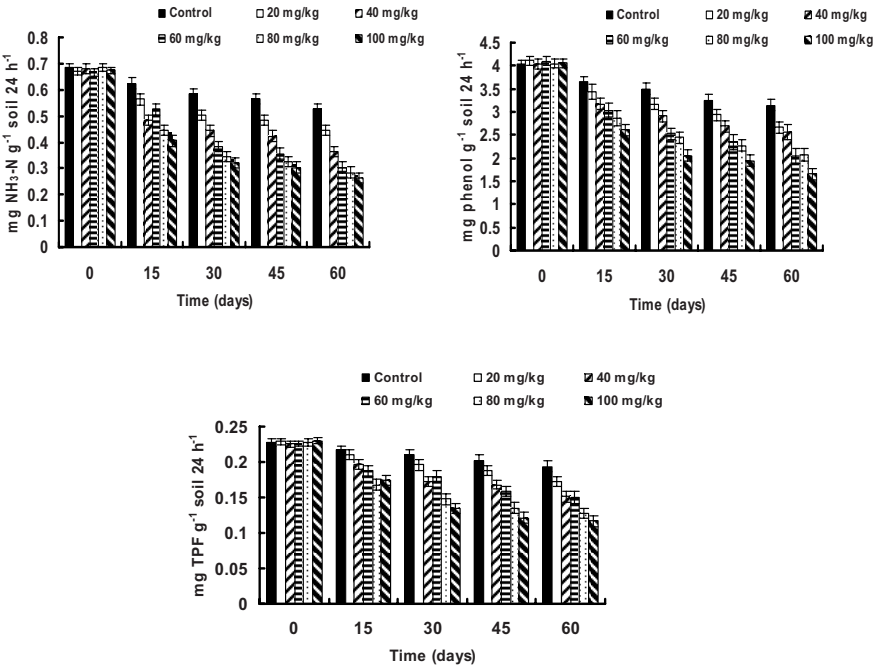

Fig. 1. Dynamics of urease, acid phosphatase and dehydrogenase activity in soil under Cd pollution (Soil urease activity is expressed as mg NH_3-N g^{-1} dry soil 24 h^{-1}, Soil phosphatase activity is expressed as the mg phenol produced g^{-1} dry soil 24 h^{-1}, Soil dehydrogenase activity is expressed as mg TPF g^{-1} dry soil 24 h^{-1}, from Akmal et al. 2005b).

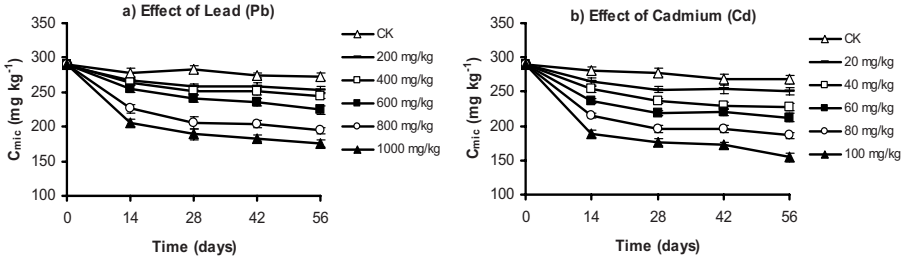

Fig. 2. Effect of Pb (**a**) and Cd (**b**) on the dynamics of soil microbial biomass carbon (C_{mic}) (from Akmal et al. 2005a).

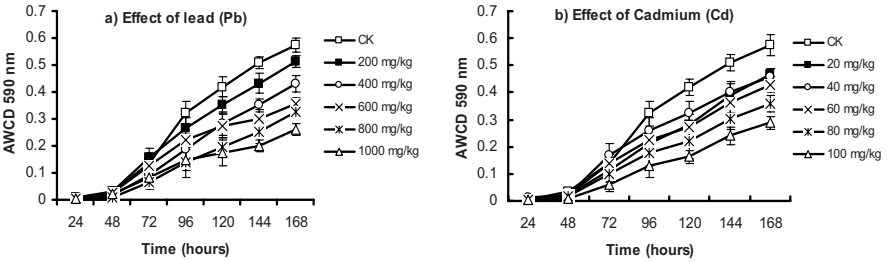

Fig. 3. Effect of Pb (**a**) and Cd (**b**) on substrate utilization efficiency of soil microbial communities as indicated by average well color development (AWCD) at 590 nm (from Akmal et al. 2005a).

Table 2. Effect of Pb and Cd interaction on soil microbial biomass carbon (mg kg^{-1}) (DAA represents days after heavy metal addition; different letters within column indicate that treatment means are significantly different at $P < 0.01$) (Authors' unpublished data, 2006)

Pb (mg kg^{-1})	Cd (mg kg^{-1})	15 DAA	30 DAA	45 DAA	60 DAA
0	0	275.7 a	270.3 a	260.3 a	250.7 a
0	20	264.3 abc	253.3 bc	254.0 a	250.3 a
0	60	236.7 e	218.7 ef	221.0 bc	212.0 b
0	100	189.0 h	176.7 gh	172.0 ef	154.3 e
200	0	267.3 ab	258.3 ab	258.7 a	253.7 a
200	20	261.0 abc	246.7 bc	224.3 b	215.3 b
200	60	249.3 cde	214.3 f	208.0 c	180.7 c
200	100	219.3 f	190.7 g	180.7 de	161.3 de
600	0	255.0 bcd	241.0 cd	235.0 b	224.7 b
600	20	260.7 abc	231.0 de	220.7 bc	214.3 b
600	60	264.3 abc	223.0 ef	223.7 b	208.7 b
600	100	240.7 de	173.7 h	164.3 f	148.0 e
1000	0	205.3 fg	189.0 g	183.3 de	175.7 cd
1000	20	236.0 e	188.7 g	191.3 d	181.7 c
1000	60	194.3 gh	144.0 i	138.0 g	121.7 f
1000	100	179.7 h	130.7 i	119.7 h	93.0 g

5 Methodological Limitations

Many studies have used soil biochemical properties as indicators of soil quality and risk assessment under heavy metal pollution, but there is still no consensus as to how they should be used. The major problems posed by the use of biochemical properties as soil quality indicators include the lack of reference values, the contradictory behavior shown by these properties when a soil is degraded, and the regional variations in expression levels. Most of these problems are derived from the scarce information available on the biochemical properties of soil. For this reason, obtaining soil quality indicators by general use of soil biochemical properties required a coordinated effort from the international scientific community to standardize the analytical methods and to compile databases of biochemical properties from soils under diverse pedo-climatic conditions and with different uses and management. Differences in sample collection, storage, pre-treatment, protocols for determining enzymatic activities (in which temperature, substrate concentration, incubation time, etc. are crucial) make it practically impossible to compare data obtained from different laboratories. Moreover, we should take into account the high degree of variability between biochemical properties, both seasonal and edaphic factors, as well as the lack of reference values or broad databases for high-quality soils that could be used to make comparisons. All this leads to the often contradictory conclusions reached by different researchers, when describing the effects of a contaminant on the soil quality. These methodological problems, along with the inherent complexities of a dynamic soil system mean that with the knowledge currently available, no estimation of soil quality, using simple indices or ratios, can be considered reliable. As Sojka and Upchurch (1999) pointed out, the use of one or two biochemical properties is not sufficient to demonstrate the complexity of the functioning of the soil system. Efforts in the use of biochemical properties as indicators of soil quality should be focused on the search for complex expressions that are capable of describing the complexity of the soil much more accurately. It is obvious that the scientific community should make greater efforts to understand the behavior of a broad group of soil properties and how they relate to each other and their role in the functioning both uncontaminated and contaminated soils.

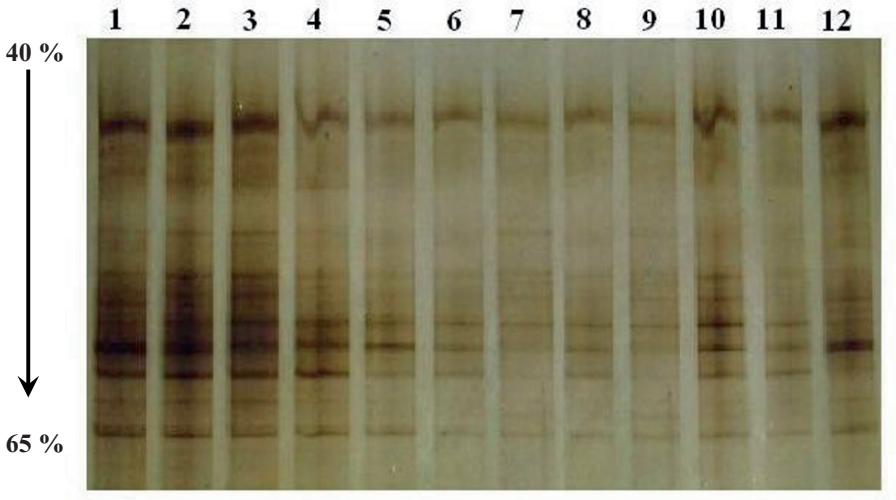

Fig. 4. DGGE profiles of amplified 16S rDNA fragments from soil with different levels of Cd pollution. Lane; 1–2 (control), 3–4 (20 mg kg^{-1} Cd), 5–6 (40 mg kg^{-1} Cd), 7–8 (60 mg kg^{-1} Cd), 9–10 (80 mg kg^{-1} Cd), and 11–12 (100 mg kg^{-1} Cd). Increasing denaturant from top (40%) to the bottom (65%) (from Akmal et al. 2005b).

6 Future Work

As many methods are currently used to monitor changes in microbial communities, there has been little work to address how each of these measures relates to one another. Unless such work is undertaken, it is unlikely that any method will be considered a reliable indicator of microbial community status. Confirmation of their applicability requires that each one should give comparable results, as a collection of methods that produce different results will not engender confidence in their use. Measurements of dehydrogenase activity are made as this approach has been shown to be applicable to many soil types and can be linked to respiration and biomass (Kelly and Tate 1998). Recent advances in molecular fingerprinting methods using signature biomarkers such as lipids and environmental nucleic acids provide a qualitative and quantitative measure of microbial diversity and community composition in undisturbed and polluted soils. The use of PCR-DGGE to determine the effect of Cd on species richness is depicted in Fig. 4. Phospholipids fatty acid (PLFA) analysis are also being performed on direct extracts from a soil detected shifts in microbial community structure depending on heavy metal concentrations, pH, moisture, organic matter content

and soil type (Baath et al. 1998; Pennanen et al. 1998). One of the problems of detecting metal toxicity to soil microorganisms and microbial processes is that the deleterious effects of heavy metal on species which perform a particular function could easily be overlooked if one species can substitute for the function performed by another. So, these methods should be used in combination with one another for comparison of microbial communities.

Most of the research work conducted in the past focused on long-term detrimental effects of metals added through sewage sludges or saw dust (Chander and Brookes 1993). At long-term field sites, soil microbial communities have had time to adapt to the stress presented by the elevated metal concentrations (Kozdroj and van Elsas 2000). Although comparison of metal-affected soil microbial communities and non-metal affected microbial communities at these sites can provide information on the changes that have occurred in the communities as a result of the metal contamination, however, such studies do not provide information on the time course of these changes (Giller et al. 1998; Pennanen 2001). Therefore, the time function of microbial communities as influenced by the extent of heavy metal contamination should be related to soil properties which are in turn resulted from soil formation processes. Interestingly, in a heavy metal toxicological study the individual microbial populations may be metal resistant, so how do microbial populations interact with each other when metals are present? Are there symbiotic relationships between metal resistant and metal sensitive populations such that the metal sensitive organism receives protection from metal toxicity while providing the metal resistant organism with some essential nutrient or carbon source? To answer such questions, we need future research. Attention should be paid to the rhizosphere under different major pedogenic processes. A number of soil microbiological properties, notably microbial biomass, basal respiration, enzymes activity and physiological profiling have been used as possible indicators of soil environmental quality, and employed in the national and international monitoring programs. The advances in molecular biological techniques are also being applied to soil ecosystem, which enable the researchers to study microbial diversity at the molecular level. But each of the above mentioned approaches offers a focus on specific aspects of soil microbiological characteristics and can give an independent analysis or changes in soil microbial community structures and functions as a result of heavy metal pollution. How to correlate and compare these approaches to obtain the standard reference value merits close attention for years to come.

Acknowledgements

The chapter was sponsored in part by Science and Technology Project of Hangzhou City (20061123B10), Science and Technology Program of Zhejiang Province (2005E10004), and the Program of Introducing Talents of Discipline to University.

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13 Changes in Antibiotic Resistance Profile of Soil Bacterial Community in Association with Land Degradation

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1 Introduction

When soil microbes function well, the soil may support plants and other lives in the ecosystem (Beare et al. 1995). Soil bacteria, as a part of the soil microbial community, may contribute to plant growth by mineral solubilization (Derylo and Skorupska 1992), nitrogen fixation (Albrecht et al. 1981), producing plant growth hormones (Neitko and Frankenberg 1989)

and supressing plant pathogens (Handman et al. 1991). If an ecosystem is subjected to a degrading impact, the soil bacterial community may then change (Frostegård et al. 1993). Deforestation caused by logging and other human activities is a serious environmental issue in the tropics. The land deprived of the original tropical forest degrades rapidly under tropical climatic conditions (Eden and Parry 1996). The land degradation results in a hard to-rehabilitate soil conditions. In such a degraded soil, the soil bacterial community profile may differ from the original one (Doi and Sakurai 2003), often accompanied by crippled soil microbial functions (Doi 2004; Jha et al. 1992; Pérez-de-Mora et al. 2006). Thus, changes in soil bacterial community profile following the elimination of a tropical vegetative type warn us of ongoing degradation of the soil ecosystem.

Methods for soil microbial community profiling have been developing (Kirk et al. 2004). Changes in soil quality can be detected by observing soil microbial aspects: fungal (Cuenca and Meneses 1996) and bacterial (Doi and Sakurai 2003) community structures, soil physiological functions (Biolog method, Garland and Mills 1991), and the distribution of biotic molecules such as respiratory quinines (Fujie et al. 1998), phospholipid fatty acids (Tunlid and White 1992), and nucleic acids (Yang et al. 2001). Profiling soil bacterial community may unveil a unique aspect of differences among soils, because the soil bacterial community profile may responds to a particular impact in a uniquely different way from the fungal community profile (Wu et al. 2008) or the physico-chemical profile (Doi and Sakurai 2003). Soil bacterial community profiles are available with the Biolog method (Garland and Mills 1991), amplified ribosomal DNA restriction analysis fingerprinting of 16S rDNA fragments (Wu et al. 2008) or counting bacterial cells that utilize single carbon sources (Doi and Sakurai 2003).

We can obtain soil bacterial community profiles by testing soil bacterial isolates' resistance patterns to single antibiotics (Brönstad et al. 1996; Doyle and Stotzky 1993; Westover et al. 1997). The insight offered by these authors is that we would be able to obtain the soil bacterial community profile by counting the number of soil bacterial cells resistant to each of multiple antibiotics. Doi (2004) tested this possibility applying the antibiotic resistance most probable number (MPN) method to soils sampled at a forest and bare ground as a result of deforestation and subsequent human activities. Then, the MPN method could discriminate the soils. The discriminatory power was comparable (Doi 2004; Doi et al. 2004) to the Biolog method (Garland and Mills 1991). However, the soils profiled in their previous work were the extremes in the area, the most fertile forest soil and the most degraded bare ground soil (Doi and Sakurai 2004). Changes in soil microbial community profile responding any impacts are

gradual, not abrupt (Frostegård et al. 1993). We were not sure how much the antibiotic resistance MPN method describes such gradual changes in soil bacterial community profile.

Thus, in this research, we tried to describe a land degradation gradient with principal components derived from data sets on antibiotic resistance profiles of soil bacterial communities over a land degradation gradient as a result of deforestation in the Sakaerat Environmental Research Station, Thailand. The antibiotic MPN method was applied in finding the impacts of land degradation on soil bacterial community profile. We tried to describe the land degradation based on differences among the antibiotic resistance profiles. We also explored relationships between soil physico-chemical characteristics and the changes in antibiotic resistance profile. The most significant soil environmental changes related to changes in the antibiotic resistance profile of soil bacterial community were then specified.

2 Materials and Methods

2.1 Site Description

The Sakaerat Environmental Research Station (SERS), Wang Nam Kiao district, Nakhon Ratchasima province, Thailand (14°30′N, 101°55′E), was established in 1967. At the time of establishment, most of the area had already been disturbed by human activities (Kaeoniam et al. 1976).

The area is 7,808 hectares and the altitude ranges from 250 to 762 m. The soil is categorized as an Orthic Acrisol according to the FAO/UNESCO scheme (FAO/UNESCO 1979). The vegetation includes dry evergreen forest (DEF), dry deciduous forest (DDF) and plantation plots as the major vegetative types (Fig. 1). The climate is classified as Aw (Köppen 1931). The annual precipitation is 1,260 mm and the average temperature is 26°C.

The DEF was primarily dominated by *Hopea ferrea* and *Shorea* spp. that formed the upper story 20–40 m above ground. A typical DEF fosters more than 1,000 trees (trunk diameter at breast height, DBH >5 cm) ha^{-1}, and the total basal area at 1.3 m height exceeded 30 m^2 ha^{-1} and the above ground biomass was over 200 tons ha^{-1} (Kanzaki et al. 1995).

The DDF was more open in comparison with the DEF and had uniformly spaced trees. The upper story, 11–35 m above ground, was formed by canopies of *Shorea obtusa*, *Pentamo suavis*, *Dipterocarpus intricatus*, *Gardenia* spp. and others. In the DDF, 875 trees (DBH>5 cm) ha^{-1} were enumerated, and the total basal area at 1.3 m height was 15 m^2 ha^{-1} and the above ground biomass was 73 tons ha^{-1} (Sahunalu and Dhanmanonda

1995). An obvious feature of the DDF was that the ground was widely covered by *Arundinaria pusilla* or *Imperata cylindrica*. Human-induced fire occurred in the DDF and burned the grass. Sometimes, the fire was strong enough to burn relatively large trees as well.

The vegetative types were distributed in a mosaic pattern in the northeastern part of the site. Bare ground (BG) without vegetation due to human activities, also scattered in the mosaic.

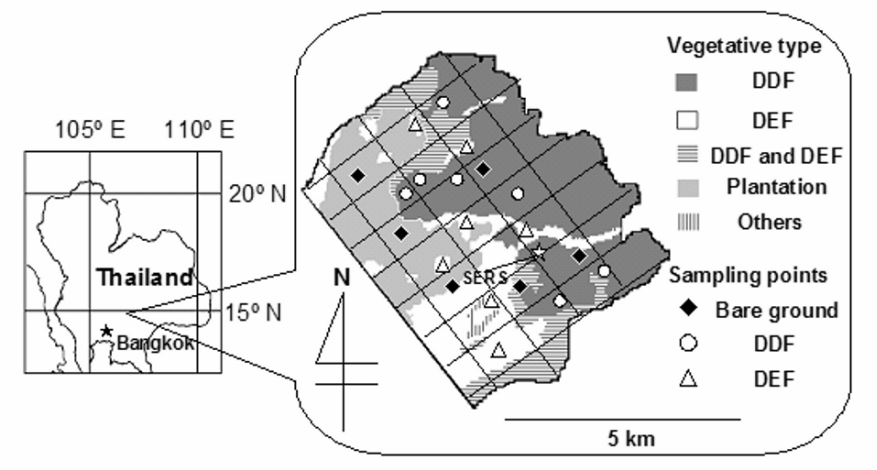

Fig. 1. The vegetative types of the Sakaerat Environmental Research Station (SERS) and the sampling points. DDF and DEF indicate dry deciduous forest and dry evergreen forest, respectively.

2.2 Soil Sampling

Soils were sampled from DEF, DDF and BG. The vegetative types were randomly distributed, and fire was thought to encourage the continuance of the fire-resistant DDF in the area (Sahunalu and Dhanmanonda 1995; Sakurai et al. 1998; Stott 1984). Thus, the vegetative mosaic was regarded as a completely randomized design (Fig. 1). The replication numbers were 7, 7 and 6 for DEF, DDF and BG, respectively. In each of the 20 grids, one vegetative type was represented (Fig. 1). All the sampling points were on slight slopes (less than 10°).

Soils were sampled on November 4, 2002. At each sampling point, a 10 m circle in diameter was set, and 6 soil cores were randomly taken from 0–5.1 cm layer by a core sampler (5 cm in diameter). Each core contained

100 mL soil. The 6 soil cores were immediately put into a plastic bag, and then mixed and passed through a 2 mm sieve.

2.3 Physico-Chemical Analyses

Soil moisture content and bulk density were determined using oven drying at 105°C for 48 h. The air-dried soils were reciprocally shaken in water (1:5 of soil to water) at room temperature for 1 h at 120 rpm to determine pH and electrical conductivity (EC). Soil organic matter (OM) was determined by the loss of ignition method. Total carbon (TC) and nitrogen (TN) were determined using a CN analyzer. Soil particle size distribution was determined with a hydrometer. Exchangeable cations (Ca, K, Mg and Na) were extracted by 1 M ammonium acetate (pH 7.0) and determined with an atomic absorption spectrophotometer. Exchangeable acidity (Al and H) was determined with titration. Cation exchange capacity (CEC) was calculated as sum of the four exchangeable cations and the exchangeable acidity. Percentage of the four exchangeable cations to CEC was regarded as the base saturation rate. Available phosphorus was determined by the Bray II method.

Values of a soil fertility index (SFI, Moran et al. 2000) or a soil evaluation factor (SEF, Lu et al. 2002) were calculated to quantify the intensity of the land degradation. SFI showed the applicability to measuring soil quality and to predicting succession rate of secondary tropical forest (Moran et al. 2000). The following equation was used to calculate SFI values (Lu et al. 2002).

$$\text{SFI} = \text{pH} + \text{OM (\%, dry soil basis)} + \text{available P (mg kg}^{-1}\text{, dry soil)} + \text{exch K (c eq kg}^{-1}\text{ dry soil)} + \text{exch Ca (c eq kg}^{-1}\text{ dry soil)} + \text{exch Mg (c eq kg}^{-1}\text{ dry soil)} - \text{exch Al (c eq kg}^{-1}\text{ dry soil)} \quad (1)$$

Possible latent drawbacks of the SFI model were pointed out by Lu et al. (2002). SFI may largely depend on pH, but an extremely high pH value is not suitable for plant growth. Moreover, pH is not an independent variable, but dependent on relative proportions of Ca, Mg and exchangeable Al in soil. Thus, they developed another index called an SEF that was calculated by the following equation.

$$\text{SEF} = [\text{exch K (c eq kg}^{-1}\text{ dry soil)} + \text{exch Ca (c eq kg}^{-1}\text{ dry soil)} + \text{exch Mg (c eq kg}^{-1}\text{ dry soil)} - \text{Log (1 + exch Al (c eq kg}^{-1}\text{ dry soil))}] \times \text{OM (\%, dry soil basis)} + 5 \quad (2)$$

Originally, SFI was developed to measure quality of soils of cacao fields (Alvim and Rosand 1974). Moran et al. (2000) extended its use in forest soils in the humid tropics, where the climate is classified as Am or Af (Köppen 1931). Recently, Doi and Sakurai (2004) found the applicability of SFI and SEF to evaluating soil quality in the SERS.

2.4 Antibiotic Resistance MPN Method

The most probable number (MPN) method developed by Wren and Venosa (1996) was used with a modification (Doi 2004). Each composite sample was profiled by this method with 3 replications for each sample. The basal medium reported by Doyle and Stotzky (1993) was slightly modified to 5.75 mM K_2HPO_4, 4.95 mM KNO_3, 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.90 mM $CaCl_2$, 1.72 mM NaCl, 12.3 μM $FeCl_3$ and 5.56 mM glucose per liter. The pH was adjusted to 6.0. Antibacterials (Lorian 1996) were chosen to profile the soils based on the MPNs of the soil bacterial communities. Final concentrations of the antibiotics were: ampicillin (2.87 mM); chloramphenicol (1.56 mM); dapson (2.02 mM); erythromycin (0.68 mM); kanamycin sulfate (34.3 μM); lasalocid (0.85 mM); nafcillin (2.42 mM); nalidixic acid (0.43 mM); neomycin•HCl (22.0 μM); novobiocin (1.89 mM); penicillin G (3.00 mM); spectinomycin•2HCl (0.25 mM); streptomycin sulfate (68.6 μM); sulfamethoxazole (0.40 mM); tetracycline (0.23 mM); and trimethoprim (1.72 mM). Ampicillin was dissolved in 1 N NH_3 solution and the pH was adjusted to 6.0. Chloramphenicol, dapson, erythromycin, lasalocid, nafcillin, nalidixic acid, novobiocin and sulfamethoxazole were dissolved in 50% (v/v) ethanol. The other antibiotics were dissolved in water. The dissolved antibiotics were filter-sterilized using a cellulose acetate membrane filter (0.20 μm, Toyo Roshi Kaisha, Ltd., Japan), then, added to the basal medium previously autoclaved and cooled to room temperature. Cycloheximide and 2,3,5-triphenyltetrazolium chloride (TTC) were filter sterilized and added to the antibiotic media at final concentrations of 0.36 and 0.20 mM, respectively. Cycloheximide was added as a fungicide. TTC which produces a deep red color in response to oxidation of the substrate was added to aid in the detection of physiological activity of the inoculated microbial communities in the wells. Tetrazolium compounds are reduced by soil bacteria, but not by soil fungi (Preston-Mafham et al. 2002). Thus, the current method profiles soil bacterial communities. The medium including no antibiotics was also prepared as the control. The media were added to microtiter plates, which were sterilized in 70% (v/v) ethanol for 30 min in advance, at 0.15 mL per well.

Ten grams of the soil sample were suspended in 90 mL of sterilized water, reciprocally shaken at room temperature for 1 h at 120 rpm. After 20 seconds, 40 mL of the upper part of the suspension was taken and centrifuged at 1,000 *g* for 5 min at 25°C. We got the soil particles at this gravity for the following reasons; (1) most soil bacterial cells attach soil particles firmly, and detaching soil bacteria from the soil particles is quite difficult (Böckelmann et al. 2003), and (2) this gravity is expected to precipitate less unknown materials than a higher gravity, then we would avoid complication in the microplate wells. The supernatant was discarded and the pellet was resuspended in 10 mL of sterilized water and diluted 10–10^7-fold with sterilized water. Fifty μL of each dilution was then added to the microtiter plate well for MPN determination, with 5 replicate wells. The inoculated plates were incubated at 28°C in the dark for 14 days, then TTC reduction was visually observed. During incubation, the plates were wrapped in a plastic film to avoid desiccation. A preliminary test using the basal medium without antibiotics resulted in no significant increase in the MPN after an incubation period longer than 14 days. In the same preliminary test, the method scored a coefficient of variance (CV) of 0.31 (n=4) after incubation for 14 days. The accuracy was comparable to that reported by Wren and Venosa (1996) who observed a CV of around 0.3 (n=5) in determination of MPNs on aromatic and aliphatic hydrocarbons. Another preliminary test was done using the basal medium containing no antibiotics as the control and the antibiotic media mentioned above. Approximately 1/100–1/10 of bacterial population from a forest soil was resistant to the antibiotics at those concentrations and the condition mentioned above. The following equation gave ratio transformed values (ter Braak and Šmilauer 1998), and standardized the raw MPNs:

$$\text{Ratio transformed value for the } i\text{-th antibiotic} = \text{MPN}i/\Sigma\text{MPN} \quad (3)$$

where, MPN*i* is the raw MPN for the *i*-th antibiotic. The transformed values were used for statistical analyses. In this paper, we call the ratio transformed value the relative abundance of resistant bacterial cells to the antibiotic.

2.5 Data Analyses

One-way analysis of variance (ANOVA) to test the significant effect of the degrading impact on each soil characteristic was performed using the computer software, SPSS 10.0.5J (SPSS Japan Inc., Tokyo). The Dunnett T3 test was chosen as the *post-hoc* test.

Principal component analysis (PCA) of the soil physico-chemical or the antibiotic resistance data set was performed with the SPSS software. Before PCA, the row MPN values were log-ratio transformed (ter Braak and Šmilauer 1998): each MPN was $\log_{10}$-transformed, then, divided by sum of the 16 log-transformed values. Simple linear regression analysis between scores on PCs based on the antibiotic resistance profiles and the soil physico-chemical characteristics was also performed using the SPSS software. To find the PCs that significantly explain variation of SFI or SEF value, multiple regression analysis between SFI or SEF values and PC scores was also performed using the SPSS software. The stepwise method at the default criteria (p=0.05 for inclusion and 0.10 for removal) was chosen.

To find the most significant soil environmental gradients associated with changes in antibiotic resistance profile, redundancy analysis (RDA) or canonical correspondence analysis (CCA, ter Braak and Šmilauer 1998) and summarizing the result as an ordination diagram were performed using CANOCO for Windows 4.02 and CanoDraw 3.10 (Microcomputer Power, NY), respectively. The RDA and the CCA are multivariate statistical techniques to relate species distribution patterns and environmental gradients in decreased dimensionality. Thus, these statistical techniques are categorized as the direct gradient analysis (ter Braak and Šmilauer 1998). The RDA detects linear species distribution patterns against a significant environmental gradient, while the CCA bell-shaped unimodal patterns (ter Braak and Šmilauer 1998). The RDA and the CCA specifies statistically more or less significant environmental gradients in relation to species distribution patterns. The significant environmental gradients are shown as vectors from the origin of the ordination diagram. Thus, significant environmental gradients and some community variables have linear (RDA) or unimodal (CCA) relationships. In the same diagram, the soil samples are located according to their scores on the ordination axes. In this research, we chose a CANOCO computation method applying the log-ratio transformation (ter Braak and Šmilauer 1998) as the above. The soil physico-chemical characteristics were used as the environmental factors. To determine the significance of each soil environmental gradient, a Monte Carlo permutation test was performed at 199 random permutations.

3 Results

3.1 Soil Physico-Chemical Characteristics and Soil Bacterial Most Probable Number On Glucose

Physico-chemical characteristics of the soils were summarized in Table 1. The values were comparable to that described in the previous reports about the SERS (Doi and Sakurai 2003; Doi et al. 2004; Sakurai et al. 1998). The one-way ANOVA indicated that most of the soil variables significantly reflected the land degradation with high values of bulk density, sand content and exchangeable acidity, and low values of moisture content, pH, OM, base (K, Ca, Mg) contents, EC, CEC, base saturation rate, TN and TC contents, available phosphorus and MPN on the glucose medium with no antibiotics. These results also told that the human activities induced several soil environmental gradients.

SFI and SEF values were summarized in Table 2. The degradation represented as the differences in vegetative type was a significant source of the variation of SFI value ($p=0.000$) that decreased as the degradation intensified. The averages for the three vegetative types were significantly different at $p=0.05$ according to the t-test. The SEF value for the DEF soil was also significantly decreased by the human-induced land degradation at $p=0.000$. The SEF value for the BG soil was significantly lower than that for the other soils, while the values for the DDF and the DEF soils did not differ significantly at $p=0.05$. It was clarified that the BG soil was the most intensively degraded soil. The SFI model showed differences between the DEF and the DDF soils more sensitively than the SEF mode. This discrimination could be attributed to pH and available phosphorus included in the SFI model. The DDF soil was shown to be an intermediate between the DEF and the BG soils, considering the variation patterns for the indexes and the single physico-chemical characteristics (Table 1).

PCA of the soil physico-chemical data provided Table 3. According to the Kaiser's criterion (Kaiser 1960), the first to fourth PCs were significant, and these four PCs explained 85% of the total variation. The first PC explained 59% of the total variation, which is comparable to the value reported in a previous report about the soils in the SERS (Doi and Sakurai 2004). For most of the characteristics, their values of eigenvectors on the first PC exceeded 0.8, but those for BD, sand content, exchangeable Al and H were negative values, lower than –0.6. As shown in a previous report (Doi and Sakurai 2004), the first PC axis was thought to indicate the general soil fertility in the SERS. The data structure was simple, having only 4 significant PCs and the first PC explained more than a half of the total variation.

3.2 Antibiotic Resistance MPN Profiles of the Soils

Antibiotic resistance profiles of the bacterial communities reflected the effects of deforestation and the land degradation (Fig. 2). The degradation was significant ($p=0.05$) as a source of variation for the numbers of soil bacterial cells resistant to lasalocid, penicillin, spectinomycin and trimethoprim, and marginally significant ($0.50 < p < 1.0$) for that to Kanamycin and streptomycin. Significant differences between two average values were observed for some antibiotics. When compared with the BG soil bacterial community, the DEF soil bacterial community had more bacterial cells resistant to dapson, kanamycin, lasalocid, nafcillin, penicillin, spectinomycin, streptomycin and trimethoprim.

These values could merely relate to the glucose-oxidizer MPN values for the soils (Table 1). To investigate if the relative abundance of resistant bacterial cells shows differences among the soils, the ratio-transformation was done (Fig. 2b). The land degradation was a significant source of variation of the relative abundance of resistant cells to ampicillin and penicillin at $p=0.05$, and to novobiocin at $p=1.0$. Significant differences between the averages were recognized. The BG soil bacterial community was rich in the relative abundance of bacterial cells resistant to ampicillin, erythromycin and novobiocin than the DEF soil bacterial community. The DEF soil bacterial community scored higher relative abundance of bacterial cells resistant to penicillin and spectinomycin, compared with the BG soil. Compared with the BG soil, the DDF soil had lower relative abundance of resistant bacterial cells to ampicillin and novobiocin, while had higher relative abundance of resistant cells to nafcillin and spectinomycin. DDF soil scored lower relative abundance of resistant bacterial cells to penicillin than DEF soil. These results showed the DEF soil bacterial community changed the original antibiotic resistance profile in the land degradation.

3.3 PCA of the Antibiotic Resistance Profiles to Find PCs that Explain the Land Degradation

Table 4 shows that seven PCs were significant according to the Kaiser's criterion (Kaiser 1960). The first PC explained 21% of the total variation, and the significant seven PCs did 80%. Several antibiotics tended to score relatively large absolute values of eigenvectors on the first PC, while they tended to score relatively small ones on the other PCs. Ampicillin, chloramphenicol, erythromycin, nalidixic acid and novobiocin scored large negative values of eigenvectors on the first axis. These results suggest that

the first PC best explained the degradation reflected on the differences in antibiotic resistance profile. The data structure was more complicated than the physico-chemical one, as shown by the larger number of significant PCs.

Simple linear regression analysis between PC scores for the antibiotic resistance data and each soil physico-chemical characteristics showed that the first PC had significant linear relationships with most of the physico-chemical characteristics (Table 5). The first PC had positive relationships with soil moisture, pH, EC, OM, CEC and several nutrients, while had negative relationships with BD and exchangeable Al and H. The sixth PC had significant positive relationships with TN, exchangeable K and Mg, and CEC. From these results, it was expected that the first PC would best explain the degradation gradient among the PCs.

Multiple regression analysis between the SFI or the SEF values and the PC scores gave the following formulae that describe the land degradation gradient based on the antibiotic resistance profiles.

$$SFI = 4.226 \times PC1\ score + 15.135\ (R = 0.559, p = 0.010) \quad (4)$$

$$SEF = 7.597 \times PC1\ score + 6.973 \times PC\ 6\ score + 21.511\ (R = 0.722, p = 0.010) \quad (5)$$

These formulae indicated that the first PC axis best explained the land degradation, and the sixth PC was a subsidiary. The high significance of the regression models shows that the PCs could describe the land degradation gradient. To visualize the degradation gradient based on the above formulae, a PC score plot was drawn (Fig. 3). Based on scores on the first and sixth PCs, each soil sample groups were located as a clump. The BG soil samples had negative scores on the first PC, while the DEF soil samples positive scores. The DDF soil samples tended to be the intermediate having higher relative similarity to the DEF soil than the BG soil.

3.4 Significant Soil Environmental Factors Related to the Changes in Antibiotic Resistance Profile

The first RDA ordination axis scored an eigenvalue of 0.493, while the first CCA axis 0.003. This contrast indicated that the linear model fitted well, but the unimodal model fitted poorly. Thus, only the RDA ordination diagram was shown in Fig. 4. The diagram visualizes the land degradation gradient. Again, the DEF and the GB soils were shown to be the extremes, while the DDF soil the intermediate. Relationships between the changes in

antibiotic resistance profile and the soil moisture or the exchangeable H gradient were found to be significant.

4 Discussions

The antibiotic resistance MPN method showed its applicability in describing the land degradation gradient. Soil biotic profiling may complement for the failure of soil physico-chemical profiling in detecting differences among soils (Kourtev et al. 2003). In other cases, soil physico-chemical profiling may find differences among soils more successfully than biotic profiling (Doi and Sakurai 2003). Therefore, multivariate data sets on soil physico-chemical and biotic characteristics do not necessarily correlate, indicating the multidimensionality of the variation of soil quality (van Straalen 2002). In this research, the most significant part of variation of antibiotic resistance profile correlated with the land degradation gradient (Formulae 4 and 5) and the soil environmental gradients (Table 5) caused by the land degradation. This is attributed to the BG, the DDF and the DEF ranked in an increasing or a decreasing order for most of the physico-chemical characteristics (Table 1) and score on the first PC for the antibiotic resistance data (Fig. 3). Both the variations for physico-chemical and antibiotic resistance profiles showed that the DDF soil was moderately disturbed (Sakurai et al. 1998), thus showed the land degradation gradient as the intermediate between the DEF and BG soils (Doi and Sakurai 2004).

The first PC derived from multivariate soil profiles does not always explain a gradient of interest. For example, the occurrence of soybean cyst caused by nematodes or the number of nematode eggs significantly correlated with the secondary and tertiary PCs derived from the soil chemical data (Francl 1993). In this research, however, the land degradation was the most decisive determinant of the changes in soil physico-chemical and antibiotic resistance profiles (Fig. 3, Table 3). This was thought to result in the strong linear correlation between the first PC for the bacterial data and the land degradation gradient. When such a decisive determinant does not exist, an abiotic environmental data set may have a more complex structure. Then, the abiotic environmental changes would result in a further complicated biotic data structure (Oline and Grant 2002). For example, yield class of Sitka spruce was best explained by the fifth PC derived from an abiotic environmental data set when MacMillan (1991) surveyed spruce stands that differed in various natural conditions.

The low eigenvalue for the CCA axis indicated few unimodal patterns against the land degradation gradient for the relative abundance of

resistant cells to the antibiotics. Rather, the variation patterns of antibiotic resistance were linear (Fig. 4). We attribute these linear variation patterns to the relatively wide adaptation by soil bacteria to environmental changes (Fenchel et al. 1998), in addition to the above decisiveness of the land degradation and the simplicity of the physico-chemical data set that should simplify the antibiotic resistance data structure (Oline and Grant 2002). The environmental gradients in Table 1 are distinctive, thus formed the distinctive land degradation gradient (Tables 2, 3). Over such distinctive environmental gradients, many plants (e.g. Wali 1999) and soil animals (e.g. Hemerik and Braussaard 2002) are expected to show unimodal distribution patterns. The linearity of the variations of antibiotic resistance patterns should be an important factor that gave the significant linear regression models (Formulae 4 and 5).

We can enlist possible factors contributed to the differences among the antibiotic resistance profiles. First, changes in soil bacterial community structure would be a factor. Doi and Sakurai (2003) profiled the soils under the DEF, the DDF and the BG with the sole carbon source MPN method (Wren and Venosa 1996). The soils had different bacterial community structures revealed by the sole carbon source MPN method. Selective forces were suggested to result in the structural changes as suggested by the lower number of soil bacterial cells in the BG soil (Table 1). Antibiotics released into soil may increase the relative abundance of resistant cells to the antibiotics, thus changes the original antibiotic resistance profile of the bacterial community (Schmitt et al. 2005). In such a case, the antibiotic is the causative agent that changes the original antibiotic resistance profile. Other selective forces, other than antibiotics' actions, are known to change the antibiotic resistance profile of soil bacterial community. Heavy metal toxicity is relatively well known to act as such a selective force (Roane and Kellogg 1996). Recently, Shrivastava et al. (2004) found that *Pseudomonas aeruginosa* isolates resistant to multiple antibiotics are rich in water treated with chlorine at a sub-optimal concentration. Genetic linkages were suggested between heavy metal resistance and antibiotic resistance (Davis et al. 2005). Therefore, various selective forces, involving no antibiotics, may alter the antibiotic resistance profile of soil bacterial community.

Table 1. Soil characteristics

Vegetative type	Moisture (%)	Bulk density (kg L^{-1})	Clay (%)	Silt (%)	Sand (%)	pH	EC (mS m^{-1})	OM (g kg^{-1} dry soil)	TN (g kg^{-1} dry soil)	TC (g kg^{-1} dry soil)	C/N
Dry evergreen forest	18.2 (1.8) [a‡]	0.97 (0.08) [b]	71.3 (9.9) [a]	15.9 (5.0) [a]	12.9 (5.6) [ab]	5.73 (0.22) [a]	11.3 [a] (2.6)	47.3 [a] (0.6)	1.83 (0.63) [a]	24.4 (8.2) [a]	13.4 (1.0) [b]
Dry deciduous forest	16.8 (2.6) [a]	1.04 (0.09) [b]	75.3 (6.9) [a]	13.1 (2.7) [a]	11.6 (6.2) [b]	5.86 (0.27) [a]	7.3 [b] (1.9)	39.9 [a] (1.0)	1.22 (0.33) [a]	21.6 (8.5) [a]	17.4 (2.0) [a]
Bare ground	6.8 (1.4) [b]	1.40 (0.10) [a]	65.2 (6.9) [a]	13.8 (3.1) [a]	21.0 (5.9) [a]	5.00 (0.22) [b]	3.1 [c] (0.7)	19.2 [b] (0.6)	0.60 (0.17) [b]	8.2 (3.0) [b]	13.5 (2.2) [b]
ANOVA[†]	0.000	0.000	0.108	0.402	0.024	0.000	0.000	0.000	0.000	0.002	0.001

Each average value is followed by the standard deviation in the parenthesis.
† The one-way ANOVA was performed hypothesizing vegetative type to be the significant source of variation. The p value for each soil characteristic is indicated.
‡ The values in the column followed by the same letter do not differ significantly at p=0.05 according to the Dunnett T3 t-test.

Table 1. (Continued)

Bray II P	K^+	Ca^{2+}	Mg^{2+}	Na^+	CEC	Exch Al	Exch H	BS	MPN on glucose
(mg L^{-1})	cmol (+) kg^{-1} dry soil							(%)	(Log_{10}MPN g^{-1} dry soil)
8.28 (2.28) [a]	0.82 (0.18) [a]	2.83 (0.88) [a]	2.94 (1.13) [a]	0.14 (0.01) [a]	7.35 (1.77) [a]	0.23 (0.07) [b]	0.37 (0.05) [b]	91 (5) [a]	7.97 (0.23) [a]
4.10 (1.67) [b]	0.55 (0.24) [ab]	2.11 (0.96) [a]	1.84 (0.72) [ab]	0.13 (0.03) [a]	5.14 (1.41) [ab]	0.36 (0.08) [a]	0.57 (0.15) [a]	80 (12) [a]	7.80 (0.24) [a]
2.83 (1.09) [b]	0.34 (0.10) [b]	0.66 (0.35) [b]	1.05 (0.69) [b]	0.14 (0.01) [a]	4.18 (0.83) [b]	0.55 (0.21) [a]	1.44 (0.65) [a]	52 (21) [b]	7.49 (0.17) [b]
0.000	0.001	0.000	0.004	0.421	0.004	0.001	0.000	0.000	0.000

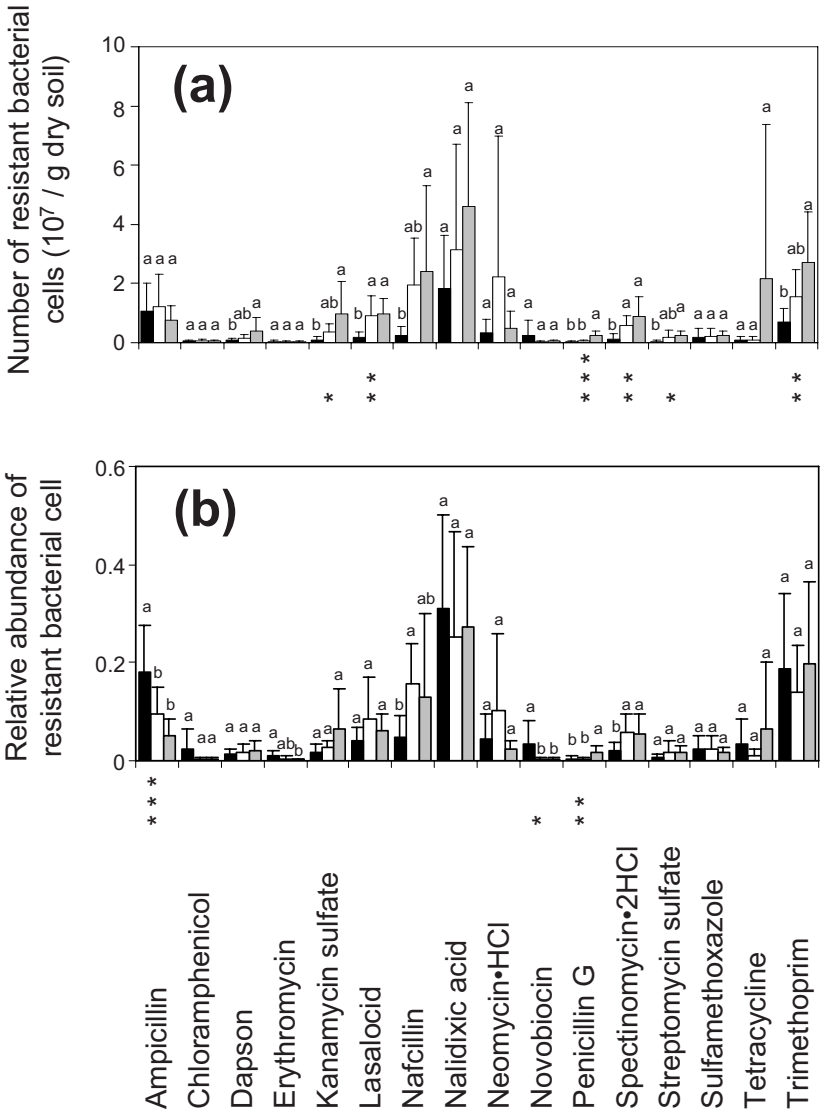

Fig. 2. Antibiotic resistance MPN profiles of the soils. The solid, open and gray bars indicate BG, DDF and DEF, respectively. The upper figure (**a**) shows the row MPNs and the lower figure (**b**) shows the ratio-transformed values. The error bar indicates the standard deviation (n=6, bare ground; n=7, dry deciduous forest or dry evergreen forest). For each antibiotic, the bars indexed with the same letter do not differ significantly at p=0.05, according to the Dunnett T3 t-test.

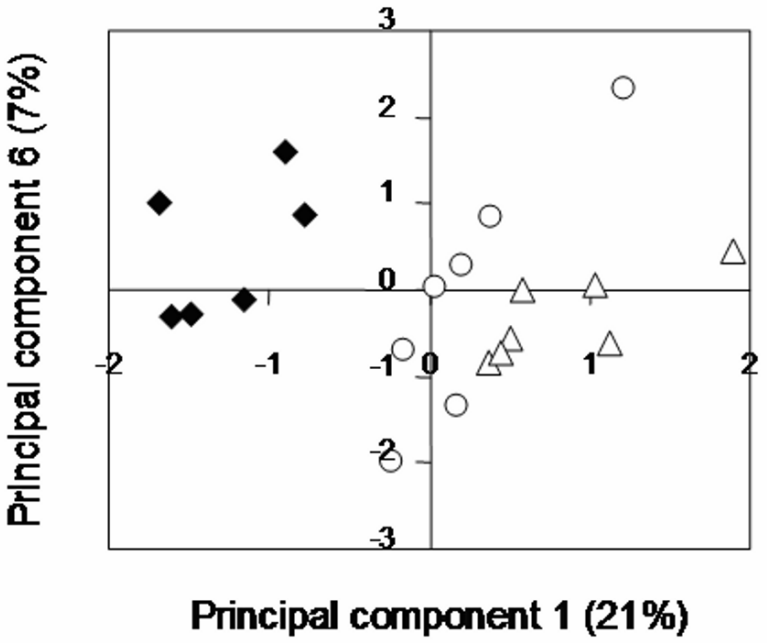

Fig. 3. Principal component score plots based on the antibiotic resistance profiles. The diamond (♦), the open square (○) and the triangle (Δ) indicate BG, DDF and DEF, respectively. The value in the parenthesis indicates the percentage of the variability explained by the principal component.

The dryness or the acidity in the degraded soil was probably the selective force that resulted in the changes in antibiotic resistance profile (Fig. 4). The decrease in soil moisture as a result of the degradation was thought to stress living things (Giuffre et al. 2001; Sakurai et al. 1998). In the SERS, soil moisture contents and numbers of viable soil bacterial cells were positively correlated (Doi and Sakurai 2003). In the land degradation, drought-susceptible soil bacteria are destroyed, while drought-tolerant bacteria survive (Kilbertus and Proth 1979). This selective process was suggested to differentiate the forest and BG soil bacterial community structures (Doi and Sakurai 2003). Then, the structural changes are likely to differentiate the antibiotic resistance profiles among the soils (Lorian 1996). In addition to the dryness, among the BG soil conditions in Table 1, acidity (Ramos et al. 1987) and high temperature due to the decreased vegetative cover and the lower moisture content (Pillai and Pepper 1991) were suggested to alter the antibiotic resistance profile of soil bacterial community as selective forces.

Table 2. Principal components, the eigenvalues, the ratios of explaining variation and the eigenvectors based on the soil physico-chemical characteristics

	Principal components (PCs)				
	PC 1	PC 2	PC 3	PC 4	PC 5
Eigenvalue	11.8	2.58	1.54	1.09	0.92
Variation explained (%)	58.7	12.9	7.7	5.4	4.6
Cumulative variation explained (%)	58.7	71.6	79.3	84.7	89.3
Moisture content	0.91	–0.01	0.13	–0.09	0.14
Bulk density	–0.87	–0.14	–0.10	0.08	–0.31
Clay	0.48	–0.75	–0.39	0.05	0.13
Silt	0.15	0.65	0.54	–0.25	–0.28
Sand	–0.67	0.59	0.19	0.07	–0.01
pH	0.86	–0.34	0.17	–0.08	–0.06
Electrical conductivity	0.86	0.19	–0.16	0.19	0.07
Organic matter	0.87	0.22	0.15	–0.02	0.34
Total N	0.90	0.24	–0.08	0.23	0.07
Total C	0.90	0.09	0.19	0.21	0.19
C/N	0.26	–0.47	0.74	–0.07	0.24
Bray II P	0.64	0.56	–0.18	–0.19	0.09
Exch K	0.90	0.16	–0.06	0.17	–0.09
Exch Ca	0.93	–0.01	–0.03	0.15	0.05
Exch Mg	0.82	0.12	–0.11	0.14	–0.42
Exch Na	0.02	0.40	–0.50	–0.56	0.31
CEC	0.83	0.27	–0.13	0.35	–0.13
Base saturation	0.87	–0.23	0.08	–0.30	–0.23
Exch Al	–0.81	0.18	0.16	0.33	0.31
Exch H	–0.83	0.23	–0.08	0.34	0.14

As the second possible factor, acquisition or loss of antibiotic resistance by bacterial cells could be important. The antibiotic resistance of bacterial cell may be genotypically (Pote et al. 2003) or phenotypically (McInroy et al. 1996) modified when the cell is subjected to environmental changes. Such changes in the bacterial cells were possibly involved in the land degradation. Releasing antibiotics into an environment often results in the resistance of bacterial cells to the antibiotics, involving genetic changes (Smalla et al. 2000). In soils, antibiotic resistance genes transfer among bacterial cells, even between different genera (Quentmeier and Friedrich 1994). Selfish DNA was pointed out to take such a role under some selective forces (Rensing et al. 2002). Moreover, such gene transfer is possible between bacterial cells and higher plants (Kay et al. 2002). Through these channels, soil bacteria may get antibiotic resistance genes.

Table 3. Soil fertility index (SFI) or soil evaluation factor (SEF) reflecting the land degradation

Vegetative type	Indexes			
	SFI		SEF	
Bare ground	6.3[c†]	±3.6	7.5[b]	±2.3
Dry deciduous forest	14.8[b]	±2.9	20.7[a]	±9.7
Dry evergreen forest	23.0[a]	±3.6	34.4[a]	±12.5
ANOVA*	0.000		0.000	

† The values in the column followed by the same letter do not differ significantly according to the Dunnett T3 t-test (p=0.05).
* Significance of vegetative type as the source of variation.

On the other hand, soil bacterial cells may lose their antibiotic resistance. Such loss may occur depending on soil environmental conditions, while independent of genetic changes (Bengtsson et al. 2004). In other cases, antibiotic resistance genes are lost from a soil bacterial community because they reduce the growth rate of the host bacteria (Rahal et al. 1998). Some antibiotic resistance genes are maintained only if the cells are continuously exposed to the antibiotics. In each soil, these mechanisms are likely to maintain the antibiotic resistance profile of its bacterial community. For example, *Pseudomonas fluorescens* produces antibiotics in soil (Cronin et al. 1997), and inoculating this bacterium into soil changes the soil microbial community structure (Johansen et al. 2005). Each soil ecosystem has antibiotic producers (Gottlieb 1976; Stevenson 1954) that associate with the soil's unique ecological structure (Beare et al. 1995). Table 1 shows the destruction of the original DEF soil ecosystem in the land degradation. It is likely that the land degradation destructed the original soil ecological structure that maintained the antibiotic resistance profile of the DEF soil. These possibly involved mechanisms are thought to be ecologically important.

Once a soil ecosystem is destructed, it takes at least some ten years to restore the original condition (Chazdon 2003). Describing the degradation gradient with the sole carbon source MPN method, Doi and Sakurai (2003) found a loss of the original diversity of the DEF soil bacterial community. This loss seemed to be responsible for losses of the original soil bacterial functions (Doi 2004), seemingly due to loss of the original functional redundancy (Lawton 1994) supported by the high structural diversity of the DEF soil bacterial community. This structurally and functionally crippled situation can be a challenge to rehabilitation of the de

graded soil in the area. In this research, the antibiotic resistance MPN method could find the differences among the soils that differ in the degree of degradation, though the fundamental causative agent of the land degradation was not any antibiotics. This indicates the possibility that the method may find changes in soil quality due to various causes. Thus, it would be worth testing this method in describing various soil-related gradients as results of various causes.

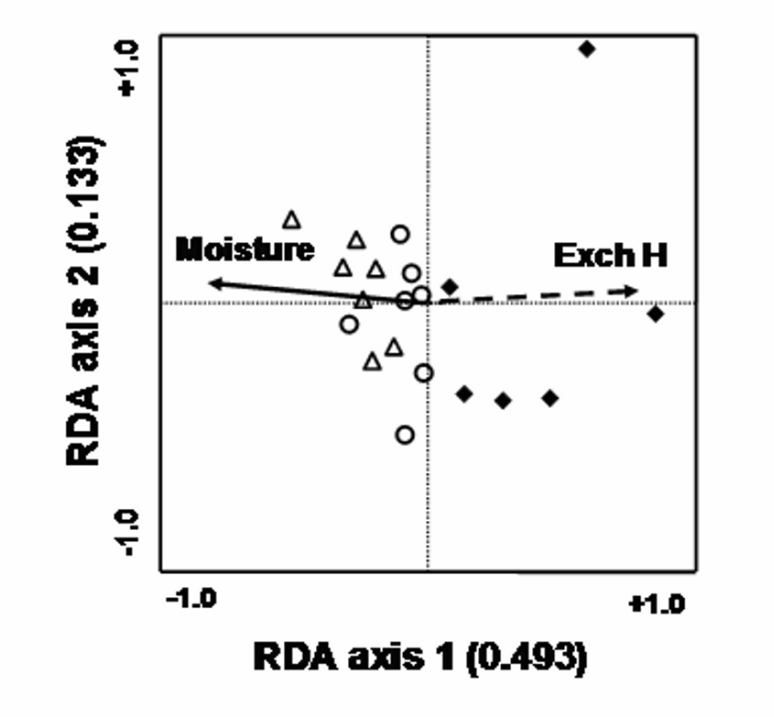

Fig. 4. Redundancy analysis ordination diagram based on the antibiotic resistance MPN data. The diamond (♦), the open square (○) and the triangle (Δ) indicate BG, DDF and DEF, respectively. The value in the parentheses indicates eigenvalue for the axis. The solid and broken arrows indicate significant environmental gradients at p=0.05 and 0.10, respectively.

Table 4. Principal components, the eigenvalues, the ratios of explaining variation and the eigenvectors based on the antibiotic resistance most probable numbers

	Principal components (PCs)							
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
Eigenvalue	3.34	2.32	1.97	1.58	1.48	1.12	1.01	0.81
Variation explained (%)	20.9	14.5	12.3	9.9	9.2	7.0	6.3	5.0
Cumulative variation explained (%)	20.9	35.4	47.7	57.6	66.8	73.8	80.1	85.2
Ampicillin	–0.34	–0.06	0.46	–0.60	0.18	–0.04	0.10	0.13
Chloramphenicol	–0.63	0.33	0.11	0.27	0.29	0.30	0.39	0.02
Dapson	0.42	–0.17	0.45	0.24	0.49	–0.25	–0.21	0.07
Erythromycin	–0.63	0.58	0.08	0.23	0.28	0.05	0.19	0.21
Kanamycin sulfate	0.33	0.34	–0.06	0.10	–0.59	0.33	0.11	0.11
Lasalocid	0.54	–0.05	0.22	0.26	0.38	–0.29	0.06	0.23
Nafcillin	0.56	0.42	–0.43	–0.24	0.26	0.08	0.13	–0.22
Nalidixic acid	–0.49	–0.20	–0.32	0.50	–0.37	–0.33	–0.23	0.14
Neomycin•HCl	–0.10	–0.13	–0.43	–0.66	0.15	–0.02	0.02	0.36
Novobiocin	–0.67	0.47	0.04	0.08	0.14	–0.09	–0.24	–0.17
Penicillin G	0.53	0.22	0.16	0.29	–0.09	0.20	0.13	0.46
Spectinomycin•2HCl	0.56	0.51	–0.39	0.15	0.19	–0.23	0.13	–0.29
Streptomycin sulfate	0.46	0.47	0.50	–0.17	–0.28	–0.01	–0.10	0.09
Sulfamethoxazole	–0.11	0.70	0.45	–0.24	–0.22	–0.18	–0.37	–0.08
Tetracycline	0.13	–0.20	0.06	0.13	0.32	0.72	–0.52	–0.09
Trimethoprim	0.02	–0.45	0.62	0.05	–0.20	0.04	0.41	–0.36

Table 5. Linear regression between principal component scores based on the antibiotic resistance MPNs and soil physico-chemical characteristics

Soil physico-chemical characteristic	Principal components (PCs) derived from the MPN data							
	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
Moisture content	***							
Bulk density	***							
Clay							*	
Silt								
Sand			**					
pH	**							
Electrical conductivity	***							
Organic matter	***							
Total N	***					**		
Total C	***							
C/N				*				
Bray II P								
Exch K	**					*		
Exch Ca	**							
Exch Mg	**					*		
Exch Na							**	
CEC	*					**		
Base saturation	***							
Exch Al	**							
Exch H	***							

***, ** and * indicate the significant linear relationship at p = 0.01, 0.05 and 0.10, respectively. The underlined asterisks indicate negative relationships.

We got the formulae for describing the land degradation gradient in the SERS. This empirical approach would contribute to conservation and rehabilitation of lands by providing formulae based on profiles of soil bacterial communities over gradients. At a site over any gradients, such a formula would predict a result on which we do not have data, if the dependent variable is a gradient of interest such as growth of any introduced plant species (MacMillan 1991). As Table 5 and formulae 4 and 5 show, a bacterial data set and soil physico-chemical characteristics would tell us the best soil condition for a particular goal (e.g. suppression of plant disease, Francl 1993). Soil bacterial community profiling is less labor-intensive than surveying plant community. The antibiotic resistance MPN method is simple and cost-effective. Once we obtain physico-chemical and antibiotic resistance data sets over a gradient of interest, depending on availability,

we can choose measuring either set of physico-chemical characteristics or antibiotic resistance MPNs in profiling another soil over the gradient for the purpose mentioned above. We can not but depend on an empirical strategy to obtain such a mathematical model (Yemefack et al. 2006). Depending on compared soils, a multivariate profiling method, take the Biolog method for example, works more (Widmer et al. 2001) or less (Waldrop et al. 2000) successfully than other profiling methods. Therefore, the antibiotic resistance MPN method must be tested in more comparative surveys before concluding the general applicability. As mentioned above, various soil environmental changes are thought to be reflected as changes in the antibiotic resistance profile of soil bacterial community. As we mentioned above, the change in antibiotic resistance profile of soil bacterial community is a unique aspect among aspects of soil quality change. It is thought to be worth considering the antibiotic resistance MPN method as a tool for monitoring soil quality changes aiming at soil conservation and rehabilitation.

Acknowledgements

Prof. Dr. Pongsak Sahunalu, Dr. Chongrak Wachrinrat and Mr. Sakhan Teejuntuk, Faculty of Forestry, Kasetsart University, Thailand helpfully provided support. Other staff members of the SERS assisted this activity. I gratefully acknowledge the support from these people.

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