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Interactions between Subsurface Microbial Assemblages and Mixed Organic and Inorganic Contaminant Systems

H.-M. Hwang,[1] J. A. Loya,[1] D. L. Perry,[2] R. Scholze[3]

[1]Department of Biology, Jackson State University,
Jackson, Mississippi 39217, USA
[2]Mail Stop 70A-1150, Lawrence Berkeley Laboratory,
Berkeley, California 94720, USA
[3]US Army Construction Engineering Research Laboratory,
Champaign, Illinois 61824-4005, USA

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Metals are introduced into aquatic environments as the results of mining and refining of ores and other human activities, such as combustion of fossil fuels, spraying of pesticides, and disposal of domestic and industrial wastes. Undoubtedly, the rapid industrial expansion and increase in domestic activities have contributed to increase in the quantities of metals being released to the environment (Collins and Stotzky 1989). Synthetic chelating agents (e.g., EDTA, DTPA, NTA, imidazole) have been used for nuclear decontamination, waste processing, and detergent industry because they react with radionuclides and heavy metal ions and form stable water-soluble complexes. Co-disposal of synthetic chelating agents with radionuclides and/or heavy metals at some landfill sites for energy and industrial wastes have caused increase in organic-inorganic mixtures transport in the subsurface environment (Bolton et al. 1990). The extensive presence and the possible persistence of these waste mixtures in those environments, therefore, underlines the need to study their effects on aquatic microbial assemblages.

Microorganisms are present at relatively high population density in surface aquatic and terrestrial environments. Microbial degradative processes have been shown to be a major removal mechanism for many organic compounds in surface waters. However, the importance of these processes in subsurface ecosystems needs more extensive studies. By comparison, the subsurface microbial transformation rates of both natural and xenobiotic chemicals are generally slower than comparable rates in soils or surface waters (Federle et al. 1986). Some chemicals such as toluene and polycyclic aromatic hydrocarbons are recalcitrant due to their resistance to microbial degradations (Wilson et al. 1982). Complete removal of xenobiotic pollutants from hazardous waste sites, however, may have to depend on degradative capability of indigenous subsurface microbial assemblages eventually. Therefore, to remediate a contaminated aquifer, the microorganisms must not be severely inhibited by the chemical pollutants. In this research, the effects of selected chemical contaminants (organic and inorganic species) on ground water bacteria and a mixture of model organic and inorganic chemicals (i.e., imidazole and copper) on surface and subsurface bacteria were determined.

Adaptation of microorganisms to in situ contaminants may be the prerequisite for microbial degradation to occur. For example, Wilson et al. (1985) reported significant biodegradation of pollutants such as naphthalene in ground water contaminated by creosote, while microbes in pristine materials from the same sites had difficulty in degrading the same compounds. In addition to adaptation, some environmental factors such as concentrations of inorganic nutrients may limit or affect biodegradation of

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Correspondence to: H. M. Hwang

subsurface organic pollutants. Availability of inorganic nutrients, such as N and P, were found to affect the length of adaptation lag periods prior to microbial transformations of xenobiotic pollutants (Lewis et al. 1986; Swindoll et al. 1988). Alternatively, anaerobic conditions may be required for initiative degradation process (e.g., dehalogenation) or complete degradation processes.

Microbial degradative activity in ground water of a chemical waste landfill site in Georgia and in ground water samples from California were the focus of this study. Bacterial numbers, microbial utilization of naturally occurring compounds such as glucose, and kinetics of microbial mineralization (aerobic and/or anaerobic processes) of several model pollutants (i.e., p-cresol and toluene) were determined. Effects of inorganic nutrients on microbial degradation of toluene, p-cresol, and phenol in ground waters were evaluated.

MATERIALS AND METHODS

Ground water samples were collected between 1989 and 1991 from a landfill site in Northeast Georgia, as described in Armstrong et al. (1991). According to chemical analyses conducted in November 1986, the dominant chemical species and their maximum concentrations in the ground water of the most contaminated sites were: manganese (43 mg/L), iron (200 mg/L), naphthalene (150 µg/L), toluene (6.9 mg/L), trichloroethylene (490 µg/L), xylene (2.3 mg/L), methylene chloride (28 mg/L), 1,1,2,2-tetrachloroethane (1.7 mg/L), formaldehyde (41 mg/L), benzene (15 mg/L), and chloroform (40 mg/L). Additional samples were collected from a ground water site in California during summer 1992. The ground water samples included control (uncontaminated) water and impacted (contaminated) water. According to chemical analyses conducted in Spring 1992, the impacted water contained dichloroethylene (81 µg/L), trichloroethylene (5.3 µg/L), and tetrachloroethylene (3.4 µg/L). Surface estuarine water was collected in July 1991 and 1992 from Berkeley Marina, Berkeley, California.

Samples of Georgia ground water were obtained from a control well (upslope) and experimental wells (downslope) from the landfill according to procedures described in Hwang et al. (1993) and Armstrong et al. (1991). In situ dissolved oxygen concentrations ranged from 11.2 to 11.5 mg/L for control water samples and 2.8 to 3.5 mg/L for impacted ground water samples. pH ranged from 5.2 to 6.2 and 6.1 to 6.5 for control well samples and impacted well samples, respectively. Temperature of the Georgia ground water samples and the California ground water samples ranged from 10.5 to 11.0°C and 15 to 20°C, respectively. pH of the California control ground water ranged from 6.1 to 6.7 and pH of the impacted ground water ranged from 6.5 to 6.9. pH of the estuarine water was 7.5 and temperature was 21±1°C. U-^{14}C-labeled p-cresol (10.33 mCi/mmol), phenol (58.2 mCi/mmol), and toluene (56.3 mCi/mmol) were obtained from Sigma Chemical Co. (St. Louis, Missouri) and D-[U-^{14}C]glucose (257.7 mCi/mmol) was obtained from DuPont Co. (Wilmington, Delaware).

Surface estuarine water and the California ground water were exposed to the model contaminants, i.e., copper, imidazole, and their complex (1:2 ratio), at varying concentrations for 17 hr before measurements of bacterial heterotrophic activities started. The effect of the model contaminants on bacterial heterotrophic activities was determined by measuring the mineralization rates (i.e., $^{14}CO_2$ production) of D-glucose. ^{14}C-glucose was added to the water samples pre-exposed to the contaminants and incubated for 5 hr. $^{14}CO_2$ resulting from glucose mineralization was collected with the two-trap method (Hwang et al. 1989), and radioactivity was measured with a liquid scintillation counter (Beckman LS 9000). pH of the California ground water was adjusted with 1 N NaOH or H_2SO_4 to 4.6 and 8.4 to assess the pH effect on microbial activity and numbers.

Procedures for kinetics measurement with the Georgia ground water samples was described in details in Hwang et al. (1989 and 1993). Ground water samples (triplicates) in this study were incubated at 24±1°C with shaking at 100 rpm. For phenol degradation experiment with the California ground water samples, ^{14}C-phenol was added at a final concentration of 0.6 µg/L. Killed controls contained formaldehyde (final concentration of 1.9%). During the incubation periods, bacterial numbers were monitored and were found to be constant. The $^{14}CO_2$ produced was collected and radioassayed as described above. Rates of toluene utilization were determined by measuring [^{14}C]-toluene uptake and mineralization (Hwang et al. 1993). Bacterial numbers in the water samples were determined by direct microscopic counting with epifluorescence microscopy of acridine orange-stained specimens (Hobbie et al. 1977). Total bacterial number in the control estuarine water, California control ground water, and impacted ground water were $(1.6\pm0.2) \times 10^7$/mL, $(1.0\pm0.1) \times 10^7$/mL, and $(1.3\pm0.2) \times 10^7$/mL, respectively. Relative rates of bacterial heterotrophic activity were determined by measuring [^{14}C]-D-glucose mineralization. [^{14}C]glucose (1 µg/L) was added to the 50-mL water samples and formalin-killed controls in 160-mL Pyrex bottles and incubated for various periods of up to 24 hr at ambient temperature (21-24°C). Glucose mineralization rates were measured by collection of $^{14}CO_2$ evolved.

To the Georgia ground water samples, aqueous stocks of NH_4NO_3 and K_2HPO_4 were added to either the control or the impacted water to assess their effects on microbial mineralization of p-cresol (10 µg/L). In another experiment with the California ground water samples, NH_4F, KNO_3, and $KHPO_4$ were added to 50 mL of the ground water in 160-mL Pyrex bottles to give N or P at final concentrations of 1 mg/L. ^{14}C-phenol was added to the bottles at a final concentration of 0.6 µg/L. Controls were the ones receiving no addition of nutrients and killed controls receiving formaldehyde at a final concentration of 1.9% (v/v).

RESULTS AND DISCUSSION

The bioavailability and toxicity of metal species to aquatic microbes depend strongly on the speciation of the metals, and other factors such as pH and concentrations of all possible ligands. The ecotoxicity of the test chemicals, i.e., copper, imidazole, and their complex, on microbes in estuarine water was determined by measurement of microbial (mainly bacterial) mineralization rates of ^{14}C-glucose at µg/L levels and total bacterial numbers. Copper (Cu^{2+}) is required in trace concentrations by microbes as a micronutrient. The chemical, physical, and toxicological properties of imidazole have not been well documented. However, in this study imidazole is used as a model chelating organic species because of this ligand's close relationship with biomolecules involving histidine residues (Bauman and Wang 1964). As indicated in Figure 1A, copper and imidazole enhanced bacterial heterotrophic activities (i.e., mineralization of glucose) up by 61% at substrate concentrations below 1 µM. Concurrent measurement of bacterial numbers, however, indicated a 30% decrease in bacterial number after exposure to 1 µM of copper (data not shown). Therefore, the enhancement of bacterial activities were assumed to exert through biochemical and physiological

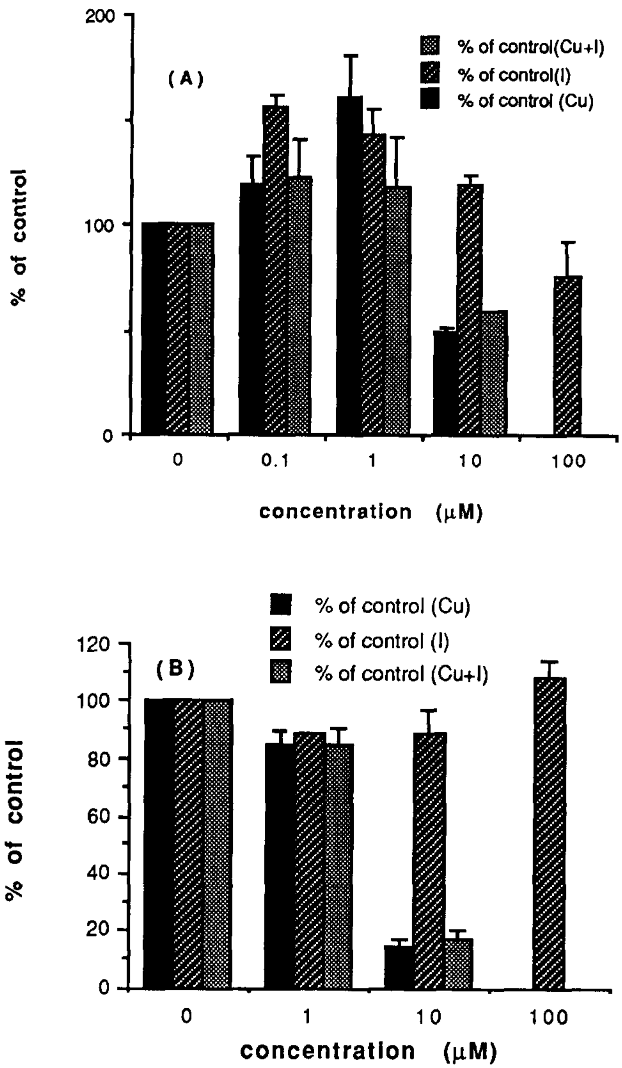

Figure 1. (A) Effects of copper (Cu^{2+}), imidazole (I), and their complex (1:2) on microbial mineralization of glucose in July estuarine water. Figure represents the mean ± 1 s.d. (B) Effects of copper (Cu^{2+}), imidazole (I), and their complex on mineralization of glucose in July California control ground water. Figure represents the mean ± 1 s.d.

mechanisms. Bacterial activities were completely inhibited by copper at the concentration of 100 μM. Nevertheless, microscopy results indicated that 44% of bacterial biomass remained in the sample after exposure to 100 μM of copper ion. The mechanisms of metal ion toxicity fall into three main categories: (1) blocking essential functional groups of biological molecules, (2) displacing an essential metal ion in biomolecules, or (3) modifying the active conformation of biomolecules (Collins and Stotzky 1989). Thus, the inhibition seemed to be mediated at molecular levels before the chemical eventually eliminated the microbial communities.

Microbial inhibition by copper at 10 μM in estuarine water was slightly relieved once copper complexed with imidazole (Cu:imidazole= 1:2 ratio). The decrease in the inhibition was assumed to be related to the decreased effective/residual concentration of copper ions. Chemical-complexation, however, often made the microscopic observation/counting of bacterial numbers very difficult, due to severe quenching of epifluorescence by the test chemicals at high concentrations.

By comparison, subsurface microbes in the California ground water samples were more susceptible to copper inhibition at the same concentration range. For example, in July control water sample, bacterial glucose mineralization activities were inhibited by 15% and 86% after exposure to Cu^{2+} at the concentration of 1 μM and 10 μM, respectively (Figure 1B); while there were 160% and 50% of mineralization activity (relative to control) in estuarine water after exposure to corresponding concentrations (Figure 1A). Concurrent chemical analysis indicated there were negligible amount of Cu^{2+} (below detection limit) in the ground water samples. The difference in response was assumed to be due to the pre-exposure of the estuary microbes to copper and/or the sequestration of copper by the dissolved salts/chelates present therein.

Drastic changes in pH can damage microorganisms by disrupting the plasma membrane and/or inhibiting the enzymatic avtivities and membrane transport proteins. After exposure to different pH treatments for 17 hr, bacterial mineralization of glucose in the California impacted ground water (pH 6.9) was inhibited by 31.5% and 1.5% at pH 4.6 and 8.4, respectively (Table 1). Apparently, microbes in the ground water system were basically neutrophiles which were more sensitive to acidic pH. Changes in the toxicity of metals occur with changes in pH. The toxicity of copper decreases as pH decreases, whereas the toxicity of nickel increases (Collins and Stotzky 1989). Such changes in toxicity are due to the effects of pH on speciation of metal species and competition between the the metal ions and hydrogen ions for binding sites on cellular

Table 1. Effect of copper and imidazole on bacterial mineralization of glucose at different pH; values are expressed as % (SD) of added [^{14}C]glucose mineralized.

	pH		
Treatment	4.6	6.9	8.4
Control (No amendment)	5.0 (0.1)	7.3 (0.2)	7.2 (0.3)
+ Copper (1 μM)	0 (0)	2.3 (0.1)	0.3 (0)
+ Copper (10 μM)	0 (0)	0.2 (0)	0.1 (0)
+ Copper (100 μM)	0 (0)	0 (0)	0 (0)
+ Imidazole (1 μM)	1.2 (0)	6.0 (0.2)	3.0 (0.1)
+ Imidazole (10 μM)	0 (0)	6.4 (0.7)	2.6 (0.5)
+ Imidazole (100 μM)	0 (0)	6.2 (0)	3.2 (0.2)
+ Copper and Imidazole (1:2 μM)	0.2 (0.2)	3.1 (1.3)	0.3 (0)
+ Copper and Imidazole (10:20 μM)	0.1 (0)	0.3 (0.1)	0.1 (0)
+ Copper and Imidazole (100:200 μM)	0 (0)	0.1 (0)	0 (0)

*[^{14}C]-D-glucose (0.1 μg/L) was added to a July California impacted ground water sample and incubated at 21°C for 5 hr after exposure to the chemicals for 17 hr.

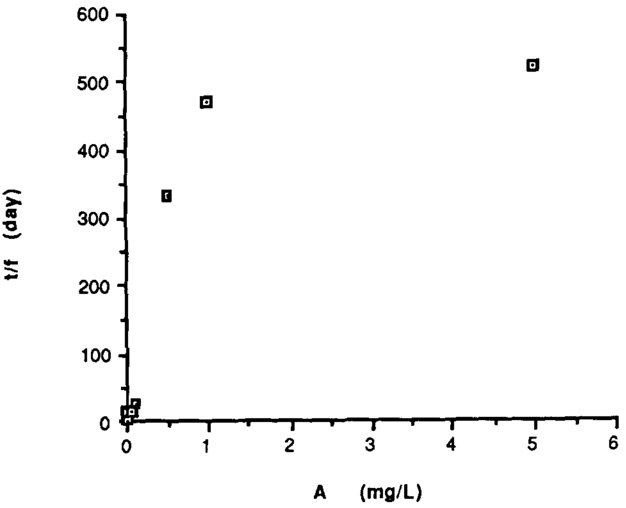

Figure 2. Wright-Hobbie plot for p-cresol mineralization in Georgia control ground water.

surface (Campbell and Stokes 1985). The toxicity of copper and imidazole seemed to increase as pH deviates from 6.9 in our study, however. Bacterial heterotrophic activity was most severely inhibited (i.e., by 100%) at pH 4.6 with additional exposure to copper between concentrations of 1 μM and 100 μM (Table 1).

The dominant copper species present at pH values of 4.6, 6.9, and 8.4 are Cu^{2+}, Cu^{2+} and $Cu(OH)^+$, and $Cu(OH)_2$, respectively. Therefore, among different copper species Cu^{2+} appeared to be the most toxic to microbial consortia in the ground water. Synergistic inhibition was seen in all cases with the exception of the treatment with copper of 1 μM at pH 4.6 (i.e., an additive inhibition). Complexation with imidazole failed to relieve the inhibition by copper in all cases. Overall, acidic pH seemed to exert more influences on microbial metabolic activity in the ground water system.

Multiphasic kinetics patterns were observed for p-cresol degradation (Figure 2) in Georgia control ground water which was similar to toluene degradation in impacted ground water from the same landfill site (Hwang et al. 1993). This is indicative of the existence of multiple uptake/degradative systems therein and such kinetic diversity had only begun to be found in ground water micobial assemblages (Hwang et al. 1993). The finding of this subsurface kinetic diversity is interesting, because it suggests bioremediation potential for the contaminated sites by engineering the subsurface indigenous microorganisms.

In addition to carbon, some inorganic elements such as nitrogen and phosphorus are needed by microbes for the synthesis of cellular material. Due to the oligotrophic habitat of most of the ground water, inorganic nutrints (e.g., N and P) commonly limit microbial activities in subsurface environments. The Georgia impacted ground water was moderately high in dissolved nitrogen and phosphorus (0.9 and 0.1 mg/L, respectively; June 1990). Nitrogen and phosphorus (0.14 mg/L PO_4^{3-}-P and 11.5 mg/L

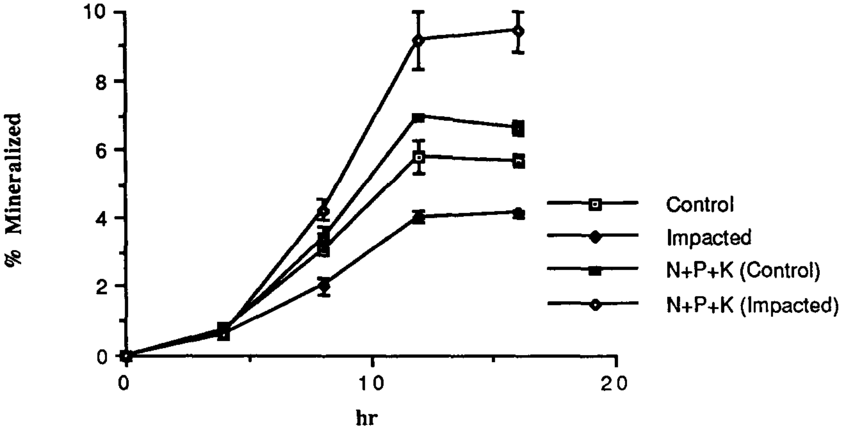

Figure 3. Effects of nutrients addition (N+P+K) on bacterial mineralization of phenol (1 µg/L) in California ground water. N & P were added at a final concentration of 1 mg/L; K was added at a final concentration of 4 mg/L.

NO_3^--N) were added to Georgia ground water of both the control and the impacted site to assess any possible effect on p-cresol degradation. No significant effect was observed, however, even when the concentration of N and P were doubled. Incubations in April 1991 for up to 4 d with the additions of ammonium nitrate, ammonium chloride, potassium nitrate and potassium chloride (up to 100 mg/L) to the impacted water also failed to stimulate microbial degradation of p-cresol. Therefore, we assume that microbial degradative activities at the landfill were not limited by depletion of these inorganic nutrients in short-term incubations. Similarly in July 1992, additions of inorganic nutrients of N (in KNO_3 or in NH_4F), K and P (in KH_2PO_4) at an elemental concentration of 1 mg/L failed to affect bacterial mineralization of phenol in the California ground water (both control and impacted water) with incubation up to 1 day. With the additions of a combination of N, P, and K (KNO_3+ KH_2PO_4; 1 mg/L of N, P, and 4 mg/L of K), however, phenol mineralization activities in the control water and in the impacted water were increased by 19% and 126%, respectively (Figure 3). The California ground water was low in N and P (below detection limit for both PO_4^{3-} and NO_3^-). Therefore, the stimulation effect of the combined-nutrients additions further confirmed that microbial degradative activities therein were limited by the depletion of inorganic nutrients. The occurrence of phenol degradative activities of the subsurface samples is interesting. Both phenol and toluene were reported as the inducers of trichloroethylene degradation, due to their induction of the production of meta-fission enzyme catechol-2,3-dioxygenase (Nelson et al. 1988). Since the impacted site was contaminated by chloroethylenes, the existence of phenol degradative activity and its enhancement by nutrients additions will have important implications with respect to the potential use of indigenous microorganisms for the remediation of the contaminated ground water.

In agreement with recent reports, our studies suggest that the composition and characteristics of the subsurface microbial assemblages may vary temporally and spacially, probably as the result of physical and chemical differences. Therefore, the subsurface systems should be sampled and studied at each site periodically to characterize the physical, chemical, and biological properties and the interactions between those components. Such investigations are required before bioremediation techniques can be actually applied to remediation of contaminated environments.

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REFERENCES

Armstrong AQ, Hodson RE, Hwang H.-M., Lewis DL (1991) Environmental factors affecting toluene degradation in ground water at a hazardous waste site. Environ Toxicol Chem 10:147-158

Bauman Jr JE, Wang JC (1964) Imidazole complexes of nickel(II), copper(II), zinc(II), and Silver(I). Inorg Chem vol. 3 no. 3:368-373

Bolton Jr H, Li SW, Workman DJ, Girvin DC (1990) Biodegradation of synthetic chelates in subsurface sediments. In: Fliermans CB, Hazen TC (eds) Proceedings of the First International Symposium on Microbiology of The Deep Subsurface. Westinghouse Savannah River Company, Aiken, South Carolina, p 4-81

Campbell PGC, Stokes PM (1985) Acidification and toxicity of metals to aquatic biota. Can J Fish Aquat Sci 42:2034-2049

Collins YE, Stotzky G (1989) Factors affecting the toxicity of heavy metals to microbes. In: Beveridge TJ, Doyle RJ (eds) Metal Ions and Bacteria. John Wiley & Sons, New York, p 31-90

Federle TW, Dobbins DC, Thornton-Manning JR, Jones DD (1986) Microbial biomass, activity, and community structure in subsurface soils. Ground Water 24:365-374

Hobbie JE, Dailey RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228

Hwang H-M, Hodson RE, Lewis DL (1989) Microbial degradation kinetics of toxic organic chemicals over a wide range of concentrations in natural systems. Environ Toxicol Chem 8:65-74

Hwang H-M, Hodson RE, Lewis DL, Scholze R (1993) Microbial degradative activity in ground water at a chemical waste disposal site. Bull Environ Toxicol Chem 50:856-863

Lewis DL, Kollig HP, Hodson RE (1986) Nutrient limitations and adaptation of microbial populations to chemical transformations. Appl Environ Microbiol 51: 598-603

Nelson MJK, Pritchard PH, Bourquin Al W (1988) Preliminary development of a bench-scale treatment system for aerobic degradation of trichloroethylene. In: Omenn GS (ed) Environmental Biotechnology: Reducing Risk from Environmental Chemicals through Biotechnology, Plenum Press, New York, p 203

Swindoll CM, Aelion CM, Pfaender FK (1988) Influence of inorganic and organic nutrients on aerobic biodegradation and on the adaptation response of subsurface microbial communities. Appl Environ Microbiol 54:212-217

Wilson JT, McNabb JF, Wilson BH, Noonan MJ. (1982) Biotransformation of selected organic pollutants in ground water. Dev Ind Microbiol 24:225-234

Wilson JT, McNabb JF, Cochran JW, Wang TH, Tomson MB, Bedient PB (1985) Influence of microbial adaptation on the fate of organic pollutants in ground water. Environ Toxicol Chem 4:721-726