INVESTIGATION OF INTERACTIONS BETWEEN AQUATIC MICROBIAL ASSEMBLAGES AND MIXED CONTAMINANT SYSTEMS

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INTRODUCTION

Metals are introduced into aquatic environments as the result 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 an increase in the quantities of metals being released to aquatic environments.[4] 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.[2] 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.

A great variety of microorganisms are present at 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. In general, the subsurface microbial transformation rates of both natural and xenobiotic chemicals are slower than comparable rates in soils or surface waters.[5] Some chemicals such as toluene, polycyclic aromatic hydrocarbons were recalcitrant due to their resistance to microbial degradations.[13] 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 an 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) in leachates 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 subsurface pollutants may be the prerequisite for microbial degradation to occur. For example, Wilson et al.[14] reported significant biodegradation of naphthalene, dibenzofuran, and 1-methylnaphthalene in ground water contaminated by creosote, while microbes in pristine materials from the same sites had difficulty in degrading these compounds. In addition to adaptation, some environmental factors such as concentrations of inorganic nutrients may limit or affect biodegradation of subsurface organic pollutants. Availability of inorganic nutrients, such as N and P, were found to affect the length of adaptation lag period prior to microbial transformations of xenobiotic pollutants.[9,11] Alternatively, anaerobic conditions may be required for the initial degradation process (e.g., dehalogenation) or complete degradation processes.[15]

Microbial degradative activity in ground water of a chemical waste landfill site in Georgia and in ground water samples provided by an energy research site in 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 also evaluated.

MATERIALS AND METHODS

Description of Study Sites

Ground water sampling was as follows: 1) samples were collected between 1989 and 1991 from a landfill site in Northeast Georgia as described in Armstrong et al.[1] 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/liter), iron (200 mg/liter), naphthalene (150 µg/liter), toluene (6.9 mg/liter), trichloroethylene (490 µg/liter), xylene (2.3 mg/liter), methylene chloride (28 mg/liter), 1,1,2,2-tetrachloroethane (1.7 mg/liter), formaldehyde (41 mg/liter), benzene (15 mg/liter), and chloroform (40 mg/liter); 2) samples were also provided by the unit of Reservoir Engineering & Hydrogeology of Earth Science Division, Lawrence Berkeley Laboratory, Berkeley, 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/liter), trichloroethylene (5.3 µg/liter), and tetrachloroethylene (3.4 µg/liter). Surface estuarine water was collected in July 1991 from Marina of the University of California at Berkeley.

Sample Collection

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.[8] and Armstrong et al.[1] Temperature and dissolved oxygen levels were measured in the wells using portable monitors (Models 33 and 57; Yellow Springs Instrument Co., Yellow Springs, OH). In situ dissolved oxygen concentrations ranged from 11.2 to 11.5 mg/liter for control water samples and 2.8 to 3.5 mg/liter for impacted ground water samples. The 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. The 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. The pH of the estuarine water was 7.5 and temperature was 21±1°C.

Chemicals

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 Company and D-[U-^{14}C]glucose (257.7 mCi/mmol) was obtained from DuPont NEN Research Products.

Effects of Copper, Imidazole, Their Complex, and pH on Microbial Assemblages

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. The compound ^{14}C-glucose was added to the water samples pre-exposed to the contaminants and incubated for 5 hr. The compounds $^{14}CO_2$ resulting from glucose mineralization were collected with the two-trap method[7], and radioactivity was measured with a liquid scintillation counter (Beckman LS 9000). The 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.

Degradation Kinetics, Microbial Biomass and Activity Measurements in Ground Water

Procedures for kinetics measurement with the Georgia ground water samples were described in details in Hwang et al.[7, 8] 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/liter.

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 measured by [^{14}C]-toluene uptake and mineralization.[8]

Bacterial numbers in the water samples were determined by direct microscopic counting with epifluorescence microscopy of acridine orange-stained specimens.[6] Total bacterial number in the control estuarine water, California control ground water, and impacted ground water were (1.6±0.2) x 10^7/ml, (1.5±0.2) x 10^6/ml, and (1.3±0.2) x 10^6/ml, respectively. Relative rates of bacterial heterotrophic activity were determined by measuring [^{14}C]-D-glucose mineralization. [^{14}C]glucose (1 µg/liter) 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 (20-24°C). Glucose mineralization rates were measured by collection of $^{14}CO_2$ evolved.

Effects of Inorganic Nutrients on Microbial Degradation of p-Cresol and Phenol

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/liter). 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/liter. The compound ^{14}C-phenol was added to the bottles at a final concentration of 0.6 µg/liter. 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

Effects of Copper, Imidazole, Their Complex, and pH on Microbial Activity and Numbers

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/liter 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. As indicated in Figure 1, copper (II) 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 the exposure to 1 µM of copper (data not shown). Therefore, the enhancement of bacterial activities were assumed to exert through biochemical and physiological mechanisms. Bacterial activities were completely inhibited by copper (II) at the concentration of 100 µM. Nevertheless, microscopy results indicated that there was 44% of bacterial biomass remaining in the sample after exposure to 100 µM of copper ion. The mechanisms of metal ion toxicity fall into three main categories: 1) blocking functional groups of biological molecules, 2) displacing an essential metal ion in biomolecules, or 3) modifying the active conformation of biomolecules.[4] Thus, the inhibition seemed to be mediated at molecular levels before the chemical eventually wiped out the microbial communities.

Microbial inhibition by copper (II) was slightly relieved once copper complexed with imidazole (Cu:imidazole= 1:2 ratio). The decrease in the inhibition was related to the decreased effective/residual concentration of copper ions. Chemical-complexation, however, sometimes 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 the 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 2); while there were 160% and 50% mineralization activity (relative to control) in estuarine water after exposure to corresponding concentrations (Figure 1). 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 sequestration of the copper ions added by salts/chelates present therein.

Drastic changes in pH can damage microorganisms by disrupting the plasma membrane and/or inhibiting the enzymatic activities and membrane transport proteins. After exposure to different pH treatments for 17 hours, bacterial mineralization of glucose in the California impacted ground water (pH 6.9) were inhibited by 31.5% and 1.5% at pH 4.6 and 8.4, respectively. Apparently, microbes in the ground water system were basically neutrophiles that were more sensitive to acidic pH. Changes in the toxicity of metals occur with changes in pH. The toxicity of Cu decreases as pH decreases, whereas the toxicity of Ni increases.[4] Such changes in toxicity are due to the effects of pH on speciation of metal species and competition between the metal ions and hydrogen ions for binding sites on cellular surface.[3] The toxicity of Cu and imidazole seemed to increase as pH decreases 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). 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.

Microbial Degradation Kinetics of p-cresol in Ground Water

Microbial degradation kinetics data were linearlized according to the method of Wright and Hobbie[16], in which concentrations of added substrate [A] were plotted vs. t/f (incubation time, t, divided by f, the fraction of substrate utilized in time t). Multiphasic kinetic patterns were observed for p-cresol degradation in Georgia control ground water as that was for toluene degradation in impacted ground water from the landfill site (Figure 3).[8] 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 [8]. The finding of this subsurface kinetic diversity is interesting because it suggests bioremediation potential for the selected chemical pollutant by subsurface indigenous microorganisms due to the flexibility of their degradative enzymatic systems. Degradation rate of toulene, a much toxic compound than p-cresol, is negligible in control ground water, however.[1] The existence of kinetic diversity of degrading toluene in impacted ground water further ensures success in the application of in situ bioremediation technique to the contamined site with adapted microbial populations.

Effects of Inorganic Nutrients on Microbial Degradation of p-cresol, toluene, and phenol

In addition to carbon, some inorganic elements are needed by microbes for the synthesis of cellular material. For example, protein synthesis requires considerable amounts of nitrogen as well as some sulfur. The synthesis of DNA and RNA also require nitrogen and some phosphorus, as does the synthesis of ATP.[12] Due to the oligotrophic habitat of most of the subsurface and ground water, foremost factors may be the influence of inorganic nutrints such as N and P, which commonly limit microbial activities in aquatic environments. The Georgia impacted ground water was moderately high in dissolved nitrogen and phosphorus then (0.9 and 0.1 mg/liter, respectively). In June 1990, nitrogen and phosphorus (0.14 mg/liter PO_4^{-3}-P and 11.5 mg/liter NO_3^--N) were added to Georgia ground water from 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 four days with the additions of ammonium nitrate, ammonium chloride, potassium nitrate, and potassium chloride (up to 100 mg/liter) to the impacted ground 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. The results of the long-term incubations, however, were different. Toluene mineralization was enhanced in impacted water amended with K and P (in KH_2PO_4), N (in NH_4Cl) or K (in KCl) with incubations up to 14 days. The increase in degradative activities were assumed to be the results of enrichment of toluene degraders by the nutrients amendments.[1]

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/liter 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/liter of N, P, and 4 mg/liter of K), however, phenol mineralization activities in the control water and in the impacted water were increased by 19% and 126%, respectively (Figure 4). 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.[10] 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 further suggest that the composition and characteristics of the subsurface microbial assemblages may vary temporally and spacially, probably as the results 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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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.

Treatment	pH		
	4.6	6.9	8.4
Control (No ammendment)	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 & Imidazole (1:2 μM)	0.2 (0.2)	3.1 (1.3)	0.3 (0)
+ Copper & Imidazole (10:20 μM)	0.1 (0)	0.3 (0.1)	0.1 (0)
+ Copper & Imidazole (100:200 μM)	0 (0)	0.1 (0)	0 (0)

*[^{14}C]-D-glucose (0.1 μg/liter) 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.

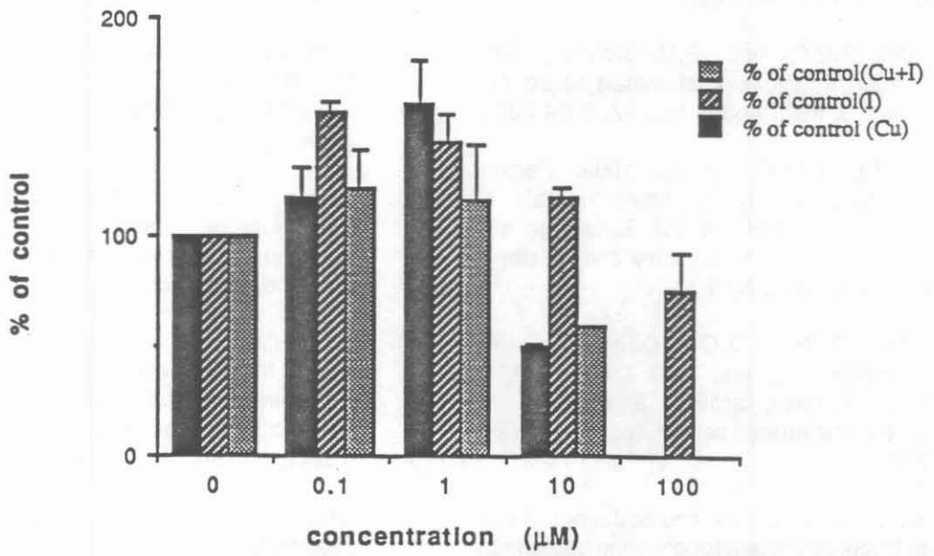

Figure 1. Effects of copper (II), imidazole, and their complex (1:2) on microbial mineralization of glucose in July estuarine water. Figure represents the mean ± 1 s.d.

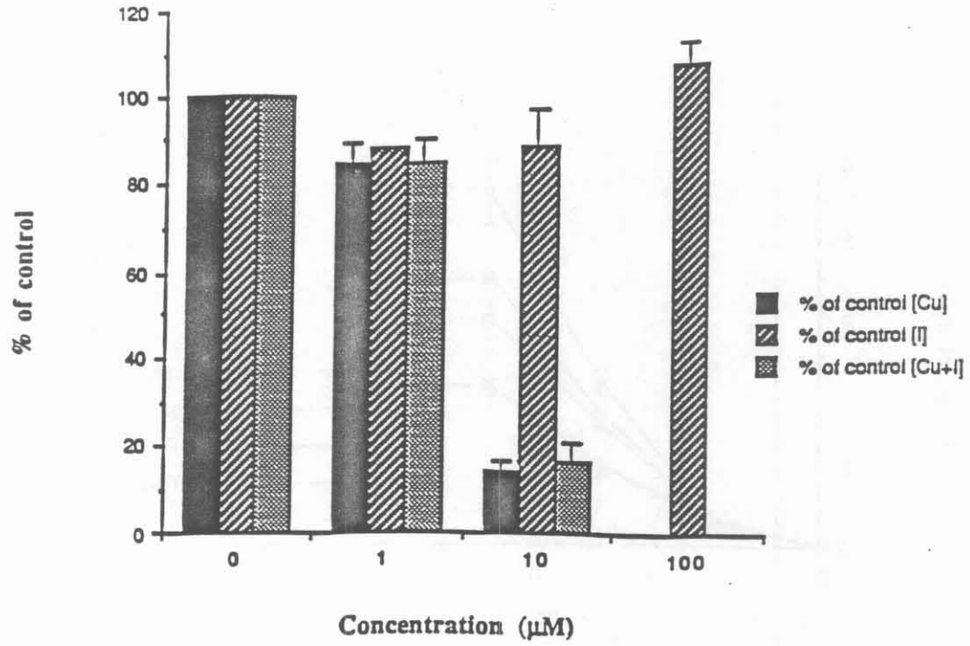

Figure 2. Effects of copper (II), imidazole, and their complex on mineralization of glucose in July California control ground water. Figure represents the mean ± 1 s.d.

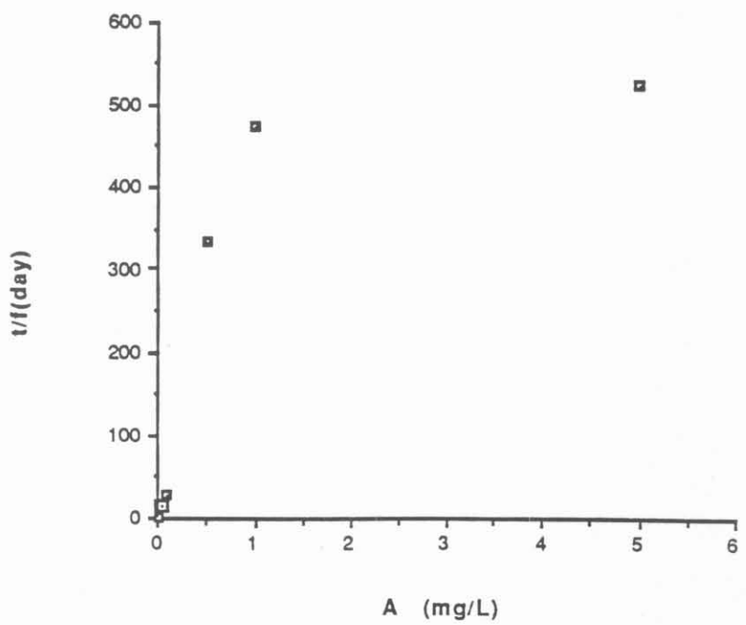

Figure 3. Wright-Hobbie plot for p-cresol mineralization in Georgia control ground water.

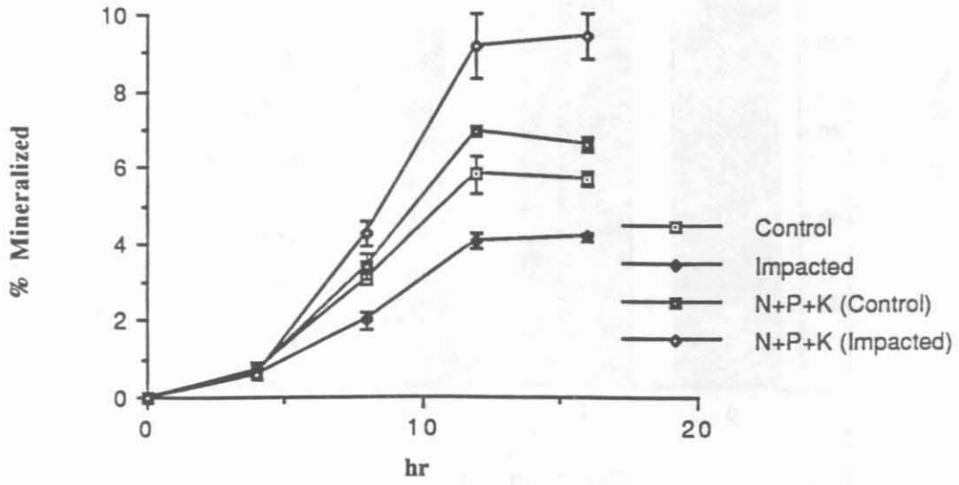

Figure 4. Effects of nutrients addition (N+P+K) on bacterial mineralization of phenol (1 µg/liter) in California ground water. N & P were added at a final concentration of 1 mg/liter; K was added at a final concentration of 4 mg/liter.