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Transformation of 3- and 4-Picoline under Sulfate-Reducing Conditions

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A microbial population which transformed 3- and 4-picoline under sulfate-reducing conditions was isolated from a subsurface soil which had been previously exposed to different *N*-substituted aromatic compounds for several years. In the presence of sulfate, the microbial culture transformed 3- and 4-picoline (0.4 mM) within 30 days. From the amounts of ammonia released and of sulfide that were determined during the transformation of 3-picoline, it can be concluded that the parent compound was mineralized to carbon dioxide and ammonia. During the transformation of 4-picoline, a UV-absorbing intermediate accumulated in the culture medium. This metabolite was identified as 2-hydroxy-4-picoline by gas chromatography-mass spectrometry and nuclear magnetic resonance analysis, and its further transformation was detected only after an additional month of incubation. The small amount of sulfide produced during the oxidation of 4-picoline and the generation of the hydroxylated metabolite indicated that the initial step in the metabolic pathway of 4-picoline was a monohydroxylation at position 2 of the heterocyclic aromatic ring. The 3- and 4-picoline-degrading cultures could also transform benzoic acid; however, the other methylated pyridine derivatives, 2-picoline, dimethylpyridines, and trimethylpyridines, were not degraded.

Pyridines, important heterocyclic compounds, are produced in large amounts as a result of industrial activity. Once pyridine derivatives enter the environment, they may persist in surface and subsurface soils for long periods (17, 18). In addition, because of their heterocyclic structure, these compounds are quite water soluble and can be transported through the soil (12) and contaminate groundwater (21). The undesirable taste and odor of heterocyclic compounds reduce the potability of groundwater (14). Furthermore, some of the pyridine derivatives can be toxic to certain organisms (20).

Anaerobic conditions can occur in water-logged or compacted soils as well as in sediments of eutrophic lakes (22). Especially when the amount of available carbon exceeds the supply of oxygen as a consequence of active microbial metabolism, anaerobic conditions can occur (4). Our current work is focused on the fate of pyridine derivatives under anaerobic conditions. Pereira et al. (16) have reported on the biotransformation of heterocyclic compounds, such as quinoline and isoquinoline, in groundwater by an indigenous consortium of microorganisms under anaerobic conditions. Similarly, other investigators (10, 18) have demonstrated transformation of alkylated pyridine derivatives in the absence of oxygen, although transformation occurred most rapidly under aerobic conditions.

Because under anaerobic conditions nitrate may not be present or may be rapidly reduced by facultative anaerobic microorganisms, we investigated the fate of 3- and 4-picoline in the presence of sulfate as an electron acceptor. Reports concerning the behavior of heterocyclic aromatic compounds under sulfate-reducing conditions are sparse. Kuhn and Suflita (12) investigated the fate of pyridine, picolines, and nicotinic acid; Brune et al. (5) reported the behavior of furfural; and Bak and Widdel (3) observed the fate of indolic compounds. Evans and Fuchs (6) concluded in their review that no complete pathway of the anaerobic metabolism of aromatic substrates by sulfate respiration has been demonstrated.

Picoline-degrading cultures were enriched from a contaminated subsurface soil which had been previously exposed to a variety of alkylated pyridine derivatives for several decades. 3-Picoline and 4-picoline were metabolized under sulfate-reducing conditions after a lag period of 2 months, whereas other methylated pyridine derivatives, such as 2-picoline and various lutidines, exhibited only minimal transformation within a 3-month incubation period (10).

MATERIALS AND METHODS

Sulfate-reducing medium. The following medium was used for growth of microorganisms under sulfate-reducing conditions (in grams per liter): NH_4Cl, 0.54; $MgCl_2 \cdot 6H_2O$, 0.41; $CaCl_2 \cdot 2H_2O$, 0.07; Na_2SO_4, 2.84; KCl, 0.18; and resazurin, 0.001. The salt solution was acidified with 0.6 ml of 2 M HCl per liter and subsequently autoclaved. The medium was then cooled under oxygen-free nitrogen gas and supplemented with the following sterile stock solutions (per liter of medium): 20 ml of 300 mM potassium phosphate (pH 7.0), 0.5 ml of trace elements, 1 ml of vitamins (11), and 2.5 ml of 100 mM sodium sulfide–10 mg of sodium dithionite–40 mg of 3- or 4-picoline. The final pH of the medium was 7.0.

Culture isolation. Microbial cultures were enriched with 3- or 4-picoline as the sole source of carbon and energy. The media were inoculated with subsurface soil (10 g of soil per 100 ml of medium) which was polluted with various pyridine derivatives from a chemical plant in Indianapolis, Ind. (10) and incubated at 28°C in the dark. Upon transformation of the substrates, the cultures (10% culture solution) were transferred to fresh medium, until all solid particles from the soil were removed.

Analytical methods. Substrates were analyzed by high-performance liquid chromatography (HPLC) (Waters Associates, Inc., Milford, Mass.), with a reverse-phase column

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(C_{18}; particle size, 4 μm; 10 cm by 5 mm; Waters Associates, Inc.). The mobile phase was a methanol-water mixture (1/1 [vol/vol]) with 0.068% triethylamine (vol/vol) buffered with 0.13% KH_2PO_4 (wt/vol) (pH 6.8). The flow rate was 1.5 ml/min. Compounds were detected by UV absorption at 254 nm (Lambda-Max 480 UV detector; Waters Associates, Inc.) and quantified with an integrator (model 3392 A; Hewlett-Packard Co., Palo Alto, Calif.) and by the external-standard method. Samples for HPLC analysis (0.5 ml) were removed periodically from the liquid phase, mixed with methanol (1/1 [vol/vol]), and stored at −20°C. Prior to injection, the samples were centrifuged at 10,000 × *g* for 10 min and the supernatants were filtered through a nylon filter (0.45-μm pore size; MSI; Fisher Scientific Co., Fairlawn, N.J.).

Compounds for proton nuclear magnetic resonance (NMR) characterization were extracted from the culture medium with dichloromethane after the cells were removed by centrifugation and filtration (as described above). After concentration of the extracts by rotary evaporation, the residue was dissolved in methanol and the compounds were separated and purified by HPLC. The collected fractions were extracted with dichloromethane, which was subsequently evaporated. The residues were dissolved in deuterated dichloromethane, and spectra were obtained on a 360-MHz Fourier transformed NMR spectrometer (model WM-360; Bruker Instruments, Cambridge, Mass.).

To isolate compounds for gas chromatography-mass spectrometry (GC-MS) analysis, the centrifuged and filtered culture medium was adjusted to pH 4.0 with 1 M HCl and extracted with ethyl acetate. Ethyl acetate was used instead of dichloromethane because the latter solvent reacted with some metabolites when analyzed by GC. After evaporation of the organic phase, the residue was dissolved in ether, and the compounds were analyzed by GC-MS (model MS-25RFA; Kratos Analytical, Ramsey, N.J.) with a capillary column (DB-5; 60 m by 0.32 mm; J&W Scientific, Folsom, Calif.) programmed from 40 to 270°C at 8°C/min. Molecular weights were confirmed by isobutane chemical-ionization MS. In order to identify different intermediates during the transformation of 4-picoline, samples for GC-MS analysis were taken at specific time intervals over several months of incubation. The amount of sulfide production during the transformation of 3- and 4-picoline was monitored by the methylene blue assay described by Gilboa-Garber (7), and the amount of ammonia was determined by the Nessler method (2). Under sterile conditions and in the absence of substrate (3- or 4-picoline), no reduction of sulfate to sulfide could be observed.

RESULTS

A microbial population that transformed 3- or 4-picoline under sulfate-reducing conditions was enriched from subsurface soil which had been contaminated with various pyridine derivatives. After disappearance of the substrate, the microbial population was transferred until a stable mixed culture was obtained.

Two mixed cultures which transformed 3-picoline (0.4 mM) or 4-picoline (0.4 mM) in the presence of sulfate within about 30 days were isolated. Both cultures were unable to metabolize the two picolines with nitrate as an electron acceptor. No transformation under anaerobic conditions in the absence of an electron acceptor occurred.

The 3-picoline-degrading culture contained at least three types of microorganisms as determined by microscopic examination. The two dominant populations in this mixed culture consisted of short rods and long rods with rounded ends, some with spores. The third population had a streptococcal shape. All of the organisms were nonmotile.

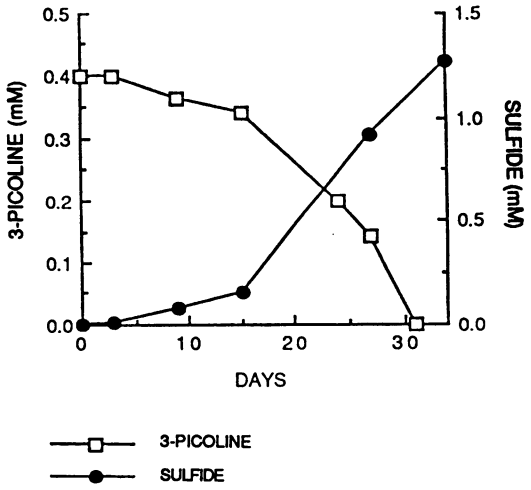


FIG. 1. Transformation of 3-picoline by a mixed culture under sulfate-reducing conditions.

The 4-picoline-degrading culture also contained three types of organisms. The dominant bacterial populations in this mixed culture were elliptic rods with pointed ends and a central spore and small, coccoid, rod-shaped organisms. The third bacterium was vibrioid shaped.

As shown in Fig. 1, incubation of 3-picoline with the 3-picoline-degrading mixed culture resulted in the rapid disappearance of the compound from the medium with a concomitant production of sulfide. The amount of sulfide formed can be predicted from the stoichiometric formula $2C_6H_7N + 8H_2O + 7SO_4^{2-} \rightarrow 12HCO_3^- + 2NH_4^+ + 7S^{2-} + 10H^+$. The agreement between the predicted and measured amounts of sulfate reduced and ammonia released into the culture medium (Table 1) indicates that 3-picoline was completely degraded to carbon dioxide and ammonia.

4-Picoline was also rapidly transformed by adapted microorganisms (Fig. 2). The 3- and 4-picoline-degrading cultures could also transform benzoate at a rate similar to that for the picolines (data not shown), but no other methylated pyridines, such as 2-picoline, dimethylpyridines, and trimethylpyridines, were metabolized (10).

However, in contrast to the results obtained with 3-picoline, only a small amount of sulfide was produced in the initial stages of 4-picoline degradation; during a 30-day incubation period, 0.4 mM 4-picoline yielded 0.084 mM sulfide while 0.4 mM 3-picoline yielded 1.26 mM sulfide (Table 1). Concurrent with the transformation of 4-picoline, a UV-absorbing product accumulated in the culture medium, as determined by HPLC analysis (Fig. 2). On the reverse-phase column, this metabolite had a shorter retention time than the parent compound, indicating that the intermediate was more polar than the parent compound, 4-picoline.

The NMR spectrum of the isolated metabolite clearly showed the methyl group as a singlet at δ 2.23. The chemical shifts and splittings of the three hydrogen atoms at positions 3, 5, and 6 on the heterocyclic aromatic ring were δ 6.38 (*m*), δ 6.12 (dd, J_1 = 6.6 Hz; J_2 = 1.6 Hz), and δ 7.25 (d, J = 6.6 Hz), respectively. The coupling pattern and chemical shifts of the aromatic-ring hydrogen atoms indicated that the

TABLE 1. Transformation of 3- and 4-picoline (0.4 mM) under sulfate-reducing conditions by a mixed culture

Substrate	Concn (mM)	Incubation (days)	Sodium sulfide			Ammonia		
			Measured (mM)	Calculated (mM)	Recovered (%)	Measured (mM)	Calculated (mM)	Recovered (%)
3-Picoline	0.4	30	1.26	1.4[a]	90	0.38	0.40[a]	94
4-Picoline	0.4	30	0.084	0.1[b]	84	0.05	0[b]	
	0.4	90	1.172	1.4[a]	84	0.36	0.4[a]	89

[a] Calculated according to the stoichiometric formula $2C_6H_7N + 8H_2O + 7SO_4^{2-} \rightarrow 12HCO_3^- + 2NH_4^+ + 7S^{2-} + 10H^+$.
[b] Calculated according to the stoichiometric formula $4C_6H_7N + 1SO_4^{2-} \rightarrow 4C_6H_7NO + 1S^{2-}$.

hydrogen atom in position 2 of the parent compound had been replaced by another substituent.

Analysis by MS indicated replacement of the hydrogen atom by a hydroxyl group (Fig. 3A). The MS spectrum of the hydroxylated derivative had a molecular ion $[M^+]$ of *m/z* 109 and an abundant fragment ion at *m/z* 80 $[M-29]^+$. This fragment could be due to a loss of a COH group. The molecular ion of the intermediate product, identified as 2-hydroxy-4-picoline, was further confirmed by chemical-ionization MS ($[MH^+] = 110$).

The mass spectrum of 2-hydroxy-4-picoline is very similar to that reported for 4-methyl-2-pyridone (Fig. 3B). In fact, 2-hydroxy-4-picoline is interconvertible with its tautomer, 4-methyl-2-pyridone, as shown in Fig. 4. It is known that 2-hydroxypyridine is subject to tautomerism involving an H interchange between the O and N atoms of the molecule (9). Thus, 2-hydroxypyridine is in equilibrium with its amide-like structure, 2-pyridone, and in polar solvents most of the 2-hydroxypyridine will be present as 2-pyridone. Therefore, it is likely that 2-hydroxy-4-picoline in the culture medium is also present as its tautomer, 4-methyl-2-pyridone.

Only small amounts of sulfide were produced during the initial stages of 4-picoline transformation. Although we cannot exclude the possibility that traces of sulfide were introduced with the inoculum, the amount of sulfide produced and the characterization of the metabolite as 2-hydroxy-4-picoline by GC-MS and NMR analysis allow us to postulate that the initial transformation of 4-picoline under sulfate-reducing conditions occurs by the following equation: $4C_6H_7N + 1SO_4^{2-} \rightarrow 4C_6H_7NO + 1S^{2-}$. After 2 months, 2-hydroxy-4-picoline was further transformed whereas sulfide accumulated. GC-MS analysis of the compounds produced during the transformation of 2-hydroxy-4-picoline had molecular ions of *m/z* 103, 123, 135, and 145. Since insufficient quantities for NMR and no reference mass spectra of the postulated intermediates were available for comparison, conclusive identification was not possible.

After 3 months of incubation, 2-hydroxy-4-picoline was completely mineralized to carbon dioxide and ammonia as determined by the amounts of sulfide and ammonia measured in the medium. Thus, the measured quantities of these products were comparable to the amounts predicted from the stoichiometric formula (Table 1). With sufficient incubation time, therefore, the adapted microorganisms were able to completely degrade both 3- and 4-picoline under sulfate-reducing conditions.

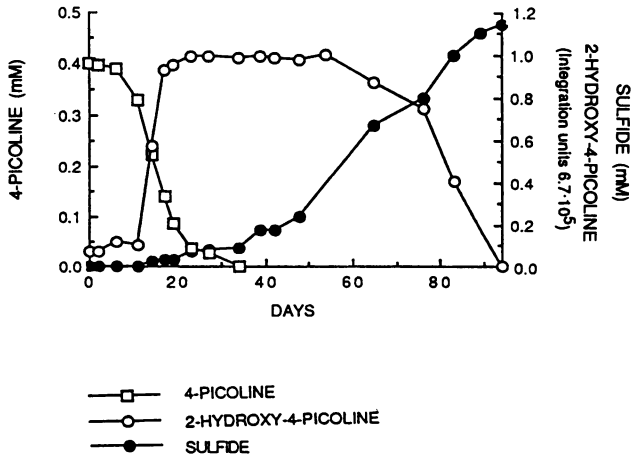


FIG. 2. Oxidation of 4-picoline by a mixed culture under sulfate-reducing conditions.

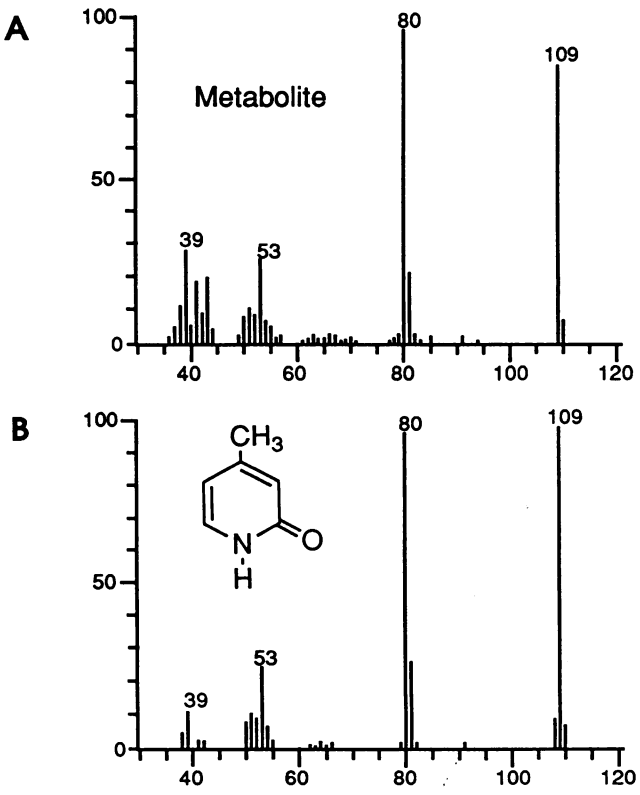
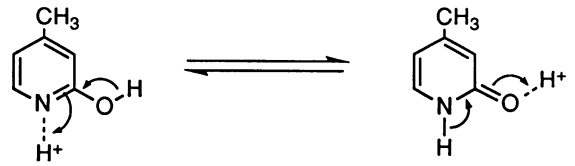


FIG. 3. Electron ionization mass spectra of a metabolite isolated from the culture medium upon transformation of 4-picoline under sulfate-reducing conditions. (A) Mass spectrum of metabolite; (B) reference mass spectrum of 4-methyl-2-pyridone (reprinted with permission of John Wiley & Sons, Inc. [15]).



2-HYDROXY-4-PICOLINE 4-METHYL-2-PYRIDONE

FIG. 4. Structural formulas of 2-hydroxy-4-picoline and its tautomer 4-methyl-2-pyridone.

DISCUSSION

From a pyridine-polluted subsurface soil, a mixed culture was isolated which could transform 3- or 4-picoline under anaerobic conditions with sulfate as an electron acceptor. During the initial transformation of 4-picoline, a UV-absorbing intermediate product accumulated in the culture medium, but no such accumulation was observed during the metabolism of 3-picoline. NMR spectroscopy and GC-MS analysis showed that 4-picoline was hydroxylated at position 2 of the aromatic ring to yield the intermediate 2-hydroxy-4-picoline.

Hydroxylation of the aromatic ring is an important reaction in the metabolism of homocyclic compounds (8). In our experiments, the rapid formation of 2-hydroxy-4-picoline indicates that monohydroxylation is most likely the first step in the transformation of 4-picoline. Additionally, the aromatic ring itself or the methyl substituent may be attacked. The culture transformed 2-hydroxy-4-picoline further within approximately 2 months. No other intermediates could be identified; therefore, beyond the hydroxylation step, the metabolic pathway of 4-picoline under sulfate-reducing conditions remains unknown.

Studies with indole under denitrifying or methanogenic conditions indicated that this substrate was hydroxylated at position 2 to form oxindole (13). Indole was also hydroxylated to oxindole by a sulfate-reducing mixed culture (19). As with 4-picoline, the preferred position for hydroxylation of indole was at position 2, adjacent to the heteroatom. Grbic-Galic (8) suggested that the steric requirements of the enzyme involved in the microbial catalysis of hydroxylation might favor substitution at position 2 and/or preclude hydroxylation at position 3 of the heterocyclic aromatic ring. In addition, electronic factors probably play an important role in the transformation of these heterocyclic compounds. Hydroxylation at position 2 destabilizes the aromatic structure and facilitates further degradation of the compound (1).

Sulfate reduction was confirmed by the detection of sulfide formation. The agreement between the expected and measured amounts of sulfide produced and the lack of transformation in the absence of the electron acceptor indicated that the transformation of the two picolines was coupled to the reduction of sulfate. The amount of sulfide measured was always slightly less than that calculated because the substrate carbon was not completely dissimilated to carbon dioxide, which indicates that a portion must have been assimilated into the biomass. In contrast to the degradation of 4-picoline, no UV-absorbing metabolites were detected during the transformation of 3-picoline. It is possible that hydroxylation of 3-picoline is the rate-limiting step in the metabolism of this compound such that, once formed, the hydroxylated derivative of 3-picoline is quickly degraded and never accumulates to detectable levels in the culture medium. Another possibility is that the pathways for the transformation of 3-picoline and 4-picoline are different. However, the fact that 3-picoline degraders were able to transform 4-picoline and vice versa suggests that 3- and 4-picoline are transformed via a similar pathway under sulfate-reducing conditions.

Thus, our data demonstrate that a microbial population derived from a pyridine-contaminated soil is capable of transforming 3- and 4-picoline under anaerobic, sulfate-reducing conditions. Furthermore, the correspondence between the measured sulfide production and that expected from stoichiometric considerations indicates that both compounds were completely degraded within 3 months. The formation of the intermediate 2-hydroxy-4-picoline suggests that monohydroxylation is an initial step in the microbial degradation of 4-picoline. Further studies are necessary to determine the subsequent metabolic pathway of these two heterocyclic aromatic compounds.

REFERENCES

1. **Acheson, R. M.** 1976. Heterocyclic analogues of benzene with one heteroatom, p. 228–297. *In* R. M. Acheson (ed.), An introduction to the chemistry of heterocyclic compounds. John Wiley & Sons, Inc., New York.
2. **American Public Health Association.** 1989. Nitrogen (ammonia): nesslerization method, p. 117–120. *In* A. E. Greenberg, R. R. Trussell, and L. S. Clesceri (ed.), Standard methods for the examination of waste and wastewater, 17th ed. American Public Health Association, Washington, D.C.
3. **Bak, F., and F. Widdel.** 1986. Anaerobic degradation of indolic compounds by sulfate-reducing enrichment cultures, and description of *Desulfobacterium indolicum* gen. nov., sp. nov. Arch. Microbiol. **146:**170–176.
4. **Bitton, G., and C. P. Gerba.** 1984. Groundwater pollution microbiology: the emerging issue, p. 1–7. *In* G. Bitton and C. P. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, Inc., New York.
5. **Brune, G., S. M. Schoberth, and H. Sahm.** 1983. Growth of a strict anaerobic bacterium on furfural (2-furaldehyde). Appl. Environ. Microbiol. **46:**1187–1192.
6. **Evans, W. C., and G. Fuchs.** 1988. Anaerobic degradation of aromatic compounds. Annu. Rev. Microbiol. **42:**289–317.
7. **Gilboa-Garber, N.** 1971. Direct spectrophotometric determination of inorganic sulfide in biological materials and in other complex mixtures. Anal. Biochem. **43:**129–133.
8. **Grbic-Galic, D.** 1990. Anaerobic microbial transformation of nonoxygenated aromatic and alicyclic compounds in soil, subsurface and freshwater sediments, p. 117–189. *In* J.-M. Bollag and G. Stotzky (ed.), Soil biochemistry, vol. 6. Marcel Dekker, Inc., New York.
9. **Joule, J. A., and G. F. Smith.** 1978. Pyridines: reactions and synthesis; oxy- and aminopyridines, p. 61–66. Heterocyclic chemistry. Van Norstrand Reinhold Co. Ltd., London.
10. **Kaiser, J.-P., and J.-M. Bollag.** 1992. Influence of soil inoculum and redox potential on the degradation of several pyridine derivatives. Soil Biol. Biochem. **24:**351–357.
11. **Kaiser, J.-P., and K. W. Hanselmann.** 1982. Fermentative metabolism of substituted monoaromatic compounds by a bacterial community from anaerobic sediments. Arch. Microbiol. **133:**185–194.
12. **Kuhn, E. P., and J. M. Suflita.** 1989. Microbial degradation of nitrogen, oxygen and sulfur heterocyclic compounds under anaerobic conditions. Studies with aquifer samples. Environ. Toxicol. Chem. **8:**1149–1158.
13. **Madsen, E. L., A. J. Francis, and J.-M. Bollag.** 1988. Environmental factors affecting indole metabolism under anaerobic conditions. Appl. Environ. Microbiol. **54:**74–78.
14. **Maya, J. A.** 1981. Pyridines in foods. J. Agric. Food Chem. **29:**895–898.
15. **McLafferty, F. W., and D. B. Stauffer.** 1989. The Wiley/NBS registry of mass spectral data. John Wiley & Sons, Inc., New York.
16. **Pereira, W. E., C. E. Rostad, D. M. Updegraff, and J. L.**

Bennett. 1987. Fate and movement of azaarenes in an aquifer contaminated by wood-treatment chemicals. Environ. Toxicol. Chem. **6**:163–176.

17. **Riley, R. G., T. G. Garland, K. Shiosaski, D. C. Mann, and R. E. Wildung.** 1981. Alkylpyridines in surface waters, groundwaters, and subsoils of a drainage located adjacent to an oil shale facility. Environ. Sci. Technol. **15**:697–701.
18. **Rogers, J. E., R. G. Riley, S. W. Li, M. L. O'Malley, and B. L. Thomas.** 1985. Microbial transformation of alkylpyridines in groundwater. Water Air Soil Pollut. **24**:443–454.
19. **Shanker, R., and J.-M. Bollag.** 1990. Transformation of indole by methanogenic and sulfate-reducing microorganisms isolated from digested sludge. Microb. Ecol. **20**:171–183.
20. **Sims, G. K., and E. J. O'Loughlin.** 1989. Degradation of pyridines in the environment. Crit. Rev. Environ. Control **19**:309–340.
21. **Stuermer, D. H., D. J. Ng, and C. J. Morris.** 1982. Organic contaminants in groundwater near an underground coal gasification site in Northwestern Wyoming. Environ. Sci. Technol. **16**:582–587.
22. **Young, L. Y.** 1984. Anaerobic degradation of aromatic compounds, p. 487–523. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.