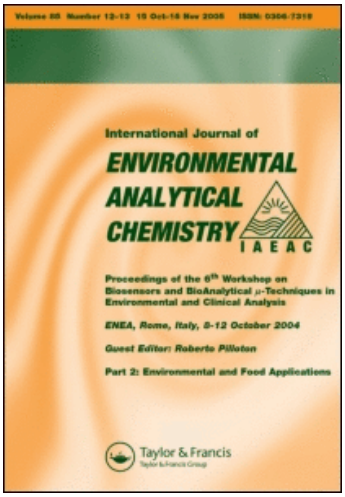


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# Biodegradation of Pyridine and Pyridine Derivatives by Soil and Subsurface Microorganisms

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# BIODEGRADATION OF PYRIDINE AND PYRIDINE DERIVATIVES BY SOIL AND SUBSURFACE MICROORGANISMS

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Large amounts of aromatic compounds are produced by various industries and two thirds of these are heterocyclic chemicals. Compared with the extensive information available on microbial degradation of homocyclic aromatic compounds, relatively little is known on the transformation and biodegradation of heterocyclic chemicals in soil. Recent concerns about the persistence of hazardous pollutants have led to a renewed interest in the biodegradation of heterocyclic compounds. Hence, we investigated the microbial degradation of pyridine and some of its alkylated derivatives under aerobic and anaerobic conditions in groundwater, subsurface sediment, and soil. Results of the investigation revealed that these compounds were degraded predominantly under aerobic conditions and, to a lesser extent, under anaerobic conditions, with nitrate or sulfate serving as electron acceptors. In groundwater polluted with various pyridine derivatives, biodegradation was limited by the absence of oxygen. Therefore, we conclude that, under appropriate conditions, bioremediation is a potentially feasible method for the clean-up of environments contaminated with heterocyclic chemicals and, in particular, pyridine derivatives.

**KEY WORDS:** Biodegradation, bioremediation, groundwater pollution, pyridine, pyridine derivatives.

## INTRODUCTION

Pyridine and pyridine derivatives comprise a group of hazardous chemicals that is used extensively in industry and agriculture. These chemicals are used as solvents and as a base for pesticides; they are also formed as a by-product of the fossil fuel processing industry. Pyridine and pyridine derivatives possess acute toxic properties and have teratogenic effects, although no known carcinogenic effects exist[1,2]. In addition, they have a very offensive odor that is detectable at low concentrations (less than 1 μg/ml). Contamination of soil, subsurface soil, and groundwater by these compounds is a severe problem because of the implications on human health and the negative impact on the potability of drinking water.

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Contamination of groundwater by pyridine and pyridine derivatives has been documented at numerous sites (Table 1). Environmental release of pyridine and its derivatives results from processes that take place during the production of fuel from coal and oil-shale, the extraction of creosote from coal-tar, the utilization of creosote in the wood-preservation industry, and the extraction of chemicals from coal-tar[2]. Pyridines can migrate quickly through the soil, as observed by Leenheer and Stuber[3]. Pyridines are relatively polar compounds that are miscible in water at very high concentrations. Therefore, these chemicals are not highly retained by mineral and organic soil fractions. Thus, any inappropriate disposal of pyridines on the soil surface will eventually result in a rapid leaching of the pollutants into the subsurface and groundwater.

To prevent health hazards and to remediate polluted sites, information on the fate of pyridine-based compounds in the environment is essential. It is particularly important to know the fate of these pollutants in the subsurface and groundwater environment. In the past few years, more research has been done on this topic. The aim of this article is to review recent developments, with emphasizing biological degradation of the compounds by soil, subsurface, and anaerobic microorganisms.

## BIODEGRADATION OF PYRIDINE AND PYRIDINE DERIVATIVES IN SOIL



Sims and Sommers[4] investigated the degradation of pyridine derivatives in surface soil under aerobic conditions. Their study demonstrated that hydroxylated, carboxylated, chlorinated, and methylated pyridine derivatives were transformed by the soil microflora. Accumulation of inorganic nitrogen during biodegradation strongly suggested that the compounds were mineralized. Biodegradability of the different chemicals was found to depend on type and position of substituent groups on the pyridine ring. Amino- and chloropyridines were the most resistant derivatives; whereas, hydroxy and carboxy derivatives were the least resistant to microbial degradation.

Another study on the degradation of pyridine derivatives in surface soil suspensions showed that increasing ring substitution tended to increase the compound's resistance to

**Table 1** Sources for pyridine and alkylpyridine contamination of groundwater and subsurface soil (adapted from ref. 8).

| *Source* | *Compounds found* | *Concentration (μg/liter)* | *Reference* |
|---|---|---|---|
| Coal gasification | Pyridine, 2-, 3-, and 4-Picoline;<br>2-, 3-, and 4- Ethylpyridine;<br>2, 4-, 2, 6-, and 3, 5-Lutidine | 51–61<br>2–10<br>1–14 | 27 |
| Wood preservation (creosote) | 2-, 3-, and 4-Picoline;<br>2, 4-, and 2 ,6-Lutidine | 100–400<br>100–2,480 | 28 |
| Coal tar distillation | 2-, 3-, and 4-Picoline;<br>2-, and 4-Ethylpyridine;<br>Phenylpyridine isomers | Not determined | 29 |
| Chemical industry | Pyridine, 2-, 3-, and 4-Picoline;<br>2-, 3-, and 4-Ethylpyridine;<br>2, 3-, 2, 4-, 2, 5-, 2, 6-, 3, 4-, and 3, 5-Lutidine<br>2-Methyl-5-ethyl, 2-Methyl-3-ethyl; and 4-Methyl-3-ethylpyridine | 6–21,500<br>6–1,390<br>13–16,400<br>5–330 | 30 |

microbial decomposition[5]. For example, a five- substituent compound, such as 4-amino-3,5,6-trichloropicolinc acid, persisted for 275 days in soil suspension. By contrast, the mono-substituent 2-picoline persisted for less than 30 days. Microbial degradation of a mixture of alkylpyridines in groundwater was examined by Rogers *et al.*[6]. It was found that the length and number of alkyl substituent groups influenced the rate of biodegradation. Degradation was found to be much faster under aerobic conditions than under anaerobic conditions.

In a recent study, Kaiser and Bollag[7] investigated the degradation of alkylpyridines in a polluted subsurface soil (Figure 1). Alkylpyridine-polluted surface and subsurface soils were obtained from a site in Indianapolis, Indiana. Unpolluted soils were obtained from the Savannah River plant site in South Carolina. In the study the degradation of various alkylpyridines was examined under different physiological conditions (i.e., aerobic versus anaerobic conditions). Degradation of the various compounds was much more extensive in the polluted soil than in the non-polluted soil. Thus, it is most likely that in the polluted soil exposure of the native microorganisms to the contaminants resulted in selection and development of alkylpyridine-degrading bacteria. In addition, anaerobic transformation was more efficient in the subsurface soil where the level of oxygen had been limited.

Kaiser and Bollag[7] also found faster degradation under aerobic conditions compared to anaerobic conditions. Experiments with the most active material from the polluted subsurface soil showed that all of the 9 tested compounds disappeared within 15 days under aerobic conditions while under anaerobic conditions only 5 compounds disappeared during this time period. Under anaerobic conditions 2- and 3-hydroxypyridine as well as 4-picoline were transformed when nitrate served as a terminal electron acceptor, whereas all the hydroxylated isomers and 3- and 4-picoline were biotransformed when sulfate was used as the terminal electron acceptor. In general the data indicated that anaerobic bacteria were less effective in degrading alkylpyridines than were aerobes.



All the above studies indicated that soil biodegradation of pyridine and pyridine derivatives was carried out mostly by aerobic microorganisms. In a study on the bioremediation of groundwater and subsurface sediment polluted with various alkylpyridines, Ronen[8] showed that mostly aerobic bioremediation occurred. Aerobic bacteria indigenous to the groundwater aquifer were able to degrade the various contaminants. Under ambient site conditions, all alkylpyridines disappeared except for 3,5-lutidine (the most persistent isomer) which was degraded after 4 weeks in slurries of sediment and groundwater incubated under aerobic conditions (Figure 2). Degradation of the different compounds depended largely on the number and position of the methyl group(s) on the pyridine ring. Compounds with a methyl group in position 3 were more resistant. This phenomenon was again observed during a column study in which subsurface sediment was leached with contaminated groundwater (Figure 3). After 5 weeks of operation, only 40% of 2,3-lutidine was removed by the column. By contrast, more than 80% of 2,4-lutidine was removed within the same time period. The subsurface sediment was used as a source of inoculum for the development of a mixed culture capable of degrading 14 different alkylpyridines. This culture, when grown in a chemostat, became very efficient in degrading the various chemicals. The efficiency of substrate removal (100 mg/l) at a dilution rate of 0.067 $day^{-1}$ was between 65% for 2,4,6-collidine and 100% for 2-picoline. The culture was very effective in degrading alkylpyridines found in polluted groundwater (Table 2). These findings suggest that, under appropriate conditions, biodegradation could serve as a biological treatment for some polluted groundwaters and aquifers.

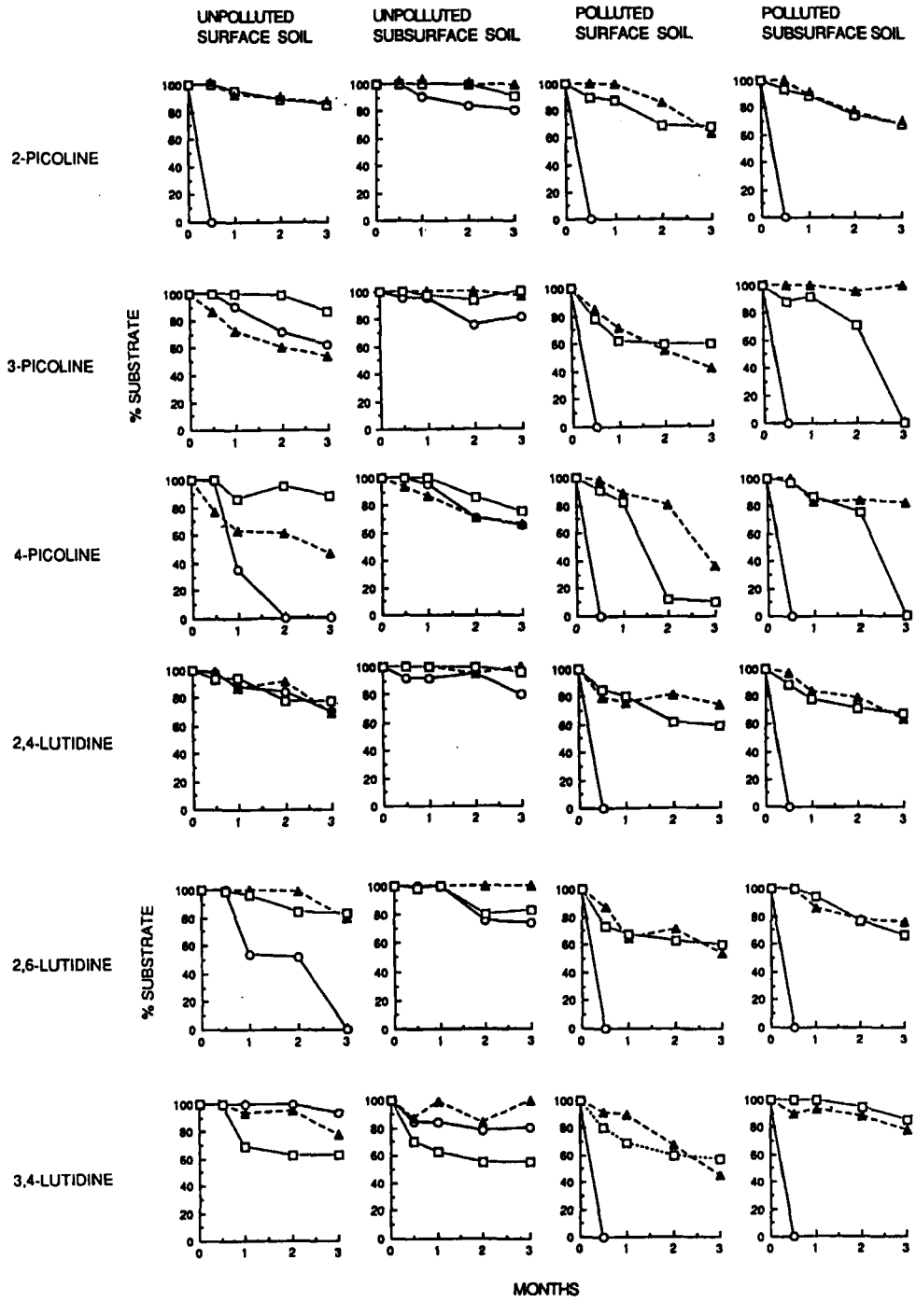


**Figure 1** Transformation of alkylpyridines by polluted surface and subsurface soils in the presence of different electron acceptors. (Values are expressed as % of initial substrate.) (adapted from ref. 7)
○——○ Aerobic conditions ▲-----▲ Denitrifying conditions □·····□ Sulfate-reducing conditions

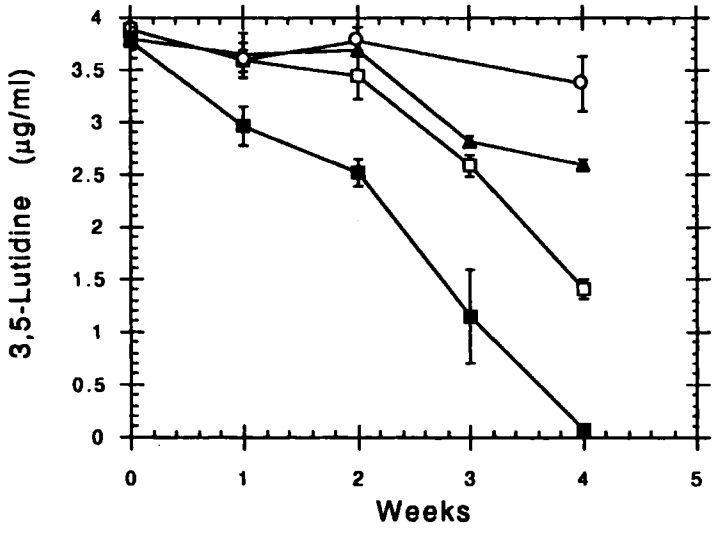


**Figure 2** Biodegradation of 3,5-lutidine in a batch culture of groundwater and subsurface sediment incubated at 15°C. The results represent the mean of 3 replicates for each treatment ± standard error (adapted from ref. 8).
- ■ - Aerobic conditions plus 5 mg/liter phosphate
- □ - Aerobic conditions (without phosphate)
- ▲ - Anaerobic conditions
- ○ - Control (250 mg $HgCl_2$/liter)



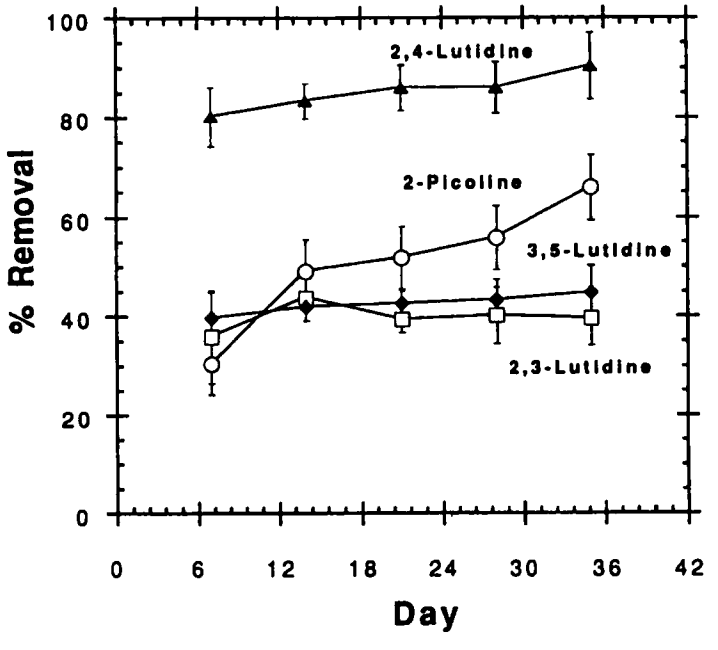


**Figure 3** Biodegradation of alkylpyridines in groundwater that leached through a column filled with polluted subsurface material. The groundwater passed through the column at a rate of 20 cm/day and the incubation was at 15°C (Adapted from ref. 8).

**Table 2** Degradation of alkylpyridines in groundwater after 4 weeks of incubation in a chemostat[a] (adapted from ref. 8).

| *Compound* | *Influent* | *Effluent* | |
|---|---|---|---|
| | | *Uninoculated* | *Inoculated*[b] |
| | | µg/liter | |
| 2-Picoline | 494 | 408 | 12 |
| 4-Picoline | 582 | 449 | 73 |
| 2-Ethylpyridine | 584 | 418 | 32 |
| 4-Ethylpyridine | 63 | 53 | 15 |
| 2,3-Lutidine | 1075 | 863 | 41 |
| 2,4-Lutidine | 2285 | 1077 | 64 |
| 2,5-Lutidine | 649 | 450 | 14 |
| 2,6-Lutidine | 1484 | 1105 | 49 |
| 3,4-Lutidine | 187 | 141 | 16 |
| 3,5-Lutidine and 2-Methyl-5-ethylpyridine | 5276 | 5075 | 42 |

[a]Dilution rate of 0.067 $day^{-1}$

[b]$1 \times 10^6$ cells/ml of a mixed culture capable of metabolizing 14 alkylpyridines



## BIODEGRADATION OF PYRIDINE AND PYRIDINE DERIVATIVES BY SOIL MICROORGANISMS

The metabolism of pyridine by aerobic microorganisms was studied in detail. Aerobic bacteria capable of growing on pyridine were isolated from soil and a few metabolic pathways determined[9–13]. Most of these studies indicated that the initial step of pyridine metabolism was a reduction of the ring to 1,4-dihydropyridine, with subsequent ring cleavage[11–13]. Inconclusive evidence for pyridine ring hydroxylation was suggested by the metabolism of pyridine by *Nocardia* sp. KM-2[9].

Aerobic bacteria capable of pyridine and alkylpyridine metabolism were also found in subsurface sediments from a polluted aquifer[8]. Five different pure cultures were isolated on 2-, 3- and 4-picoline and 2,4-, and 2,6-lutidine. Each of the isolated bacteria was able to use the respective substrate as a sole carbon and nitrogen source (Figure 4). In addition, the different cultures had similar morphological properties, but the substrate specificity of the various isolates was very distinct (Table 3).

Figure 5 describes the metabolic pathway of pyridine by aerobic bacteria. Pathway A shows pyridine metabolism by *Bacillus* 4[13]. The metabolism of pyridine by *Nocardia* KM-2 is described by pathway B, where the initial degradation reaction involves hydroxylation of the ring and formation of 3-hydroxypyridine. Subsequently, both degradation pathways are identical. After cleavage of the aromatic ring to unstable hypothetical intermediates (in brackets), hydrolysis of the carbon-nitrogen bond liberates formamide and succinate semialdehyde. The former is hydrolyzed to form formate and ammonia, while the latter product, succinate semialdehyde, undergoes oxidation to form succinic acid, which enters the metabolic pool.

Degradation of 2-picoline by *Arthrobacter* sp. involved ring reduction without the formation of stable aromatic intermediates[14]. By contrast, metabolism of 3-picoline by *Pseudomonas* sp. KM-3 consisted of hydroxylation of the methyl group and subsequent formation of the corresponding carboxylic acid[9]. Biotransformation of 2,6-lutidine by several different microorganisms was initiated out via *N*-oxidation of the compound[14].

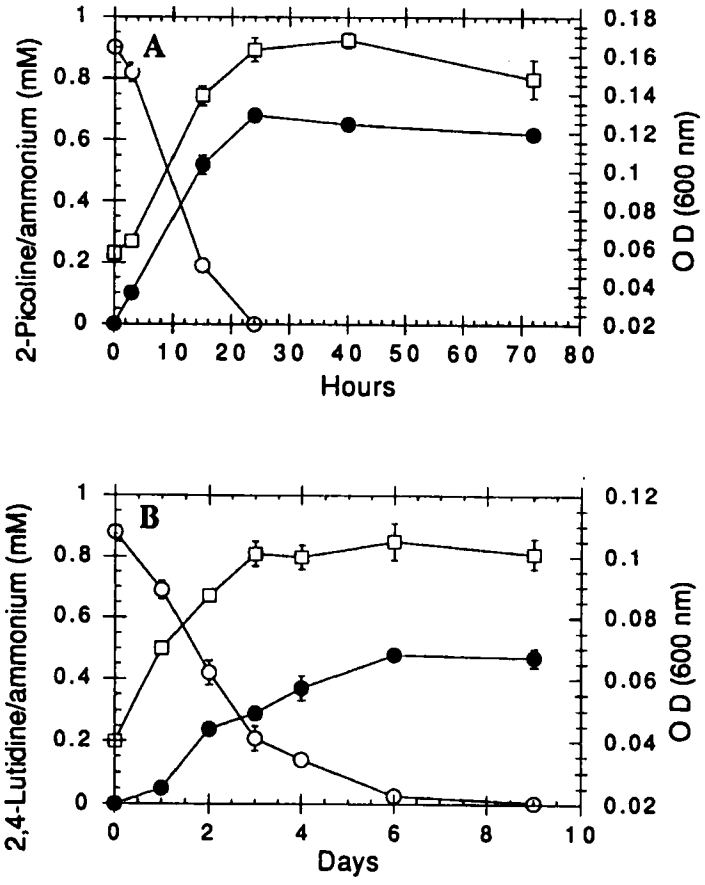


**Figure 4** Degradation of 2-picoline (A) and 2,4-lutidine (B) by the pure cultures isolated from the contaminated subsurface sediment. Results are the mean of 3 replicates for each culture ± standard error (adapted from ref. 8).
- ○ - Substrate (2-picoline or 2,4-lutidine) - ● - Optical density (growth)
- □ - Ammonium released into the medium

*Nocardia* sp. 279, *Brevibacterium* sp.[15], and *Candida tropicalis* transformed 2,6-lutidine, 2-picoline, and 3-picoline to the corresponding *N*-oxide while hexadecane served as the main carbon source[15]. Hydroxylation of methyl groups by resting cells of the fungus *Sporotrichum sulfurescens* resulted in the formation of 2-methyl-6-hydroxymethylpyridine and 2,6-*bis*(hydroxymethyl)pyridine[16].

Unlike the relatively ample knowledge available on the degradation of pyridine compounds by aerobic organisms, little is known about the microbial metabolism of pyridine under anaerobic conditions[2,17]. This aspect of microbial activity is important because subsurface and groundwater environments (where pollutants persist for a long time) are often low in oxygen. Indeed, in the few studies that examined disappearance of pyridine compounds under anaerobic conditions, no axenic anaerobic culture capable of growing on pyridine was isolated and no metabolic pathway suggested[2]. However, recent studies by Kaiser and Bollag[18] revealed that the pyridine ring can be mineralized anaerobically by nitrate- and sulfate-reducing microbial populations.

**Table 3** Substrate specificity of different pure cultures of bacteria isolated from a polluted subsurface sediment (adapted from ref. 8).

| *Compound* | *2-Picoline degrader* | *3-Picoline degrader* | *4-Picoline degrader* | *2,4-Lutidine degrader* | *2,6-Lutidine degrader* |
|---|---|---|---|---|---|
| Pyridine | – | – | ++ | ++ | – |
| 2-Picoline | +++ | ++ | ++ | – | – |
| 3-Picoline | – | +++ | – | – | – |
| 4-Picoline | – | – | +++ | ++ | – |
| 2-Ethylpyridine | +++ | ++ | ++ | – | – |
| 3-Ethylpyridine | – | +++ | – | – | – |
| 4-Ethylpyridine | – | – | ++ | ++ | – |
| 2,3-Lutidine | – | – | – | – | – |
| 2,4-Lutidine | – | – | ++ | +++ | – |
| 2,5-Lutidine | – | – | ++ | ++ | – |
| 2,6-Lutidine | – | – | – | – | +++ |
| 3,4-Lutidine | – | – | – | – | – |
| 3,5-Lutidine | – | – | – | – | – |
| 2,4,6-Collidine | – | – | – | – | +++ |

– No growth
\+ Little growth
++ Moderate growth
+++ Extensive growth



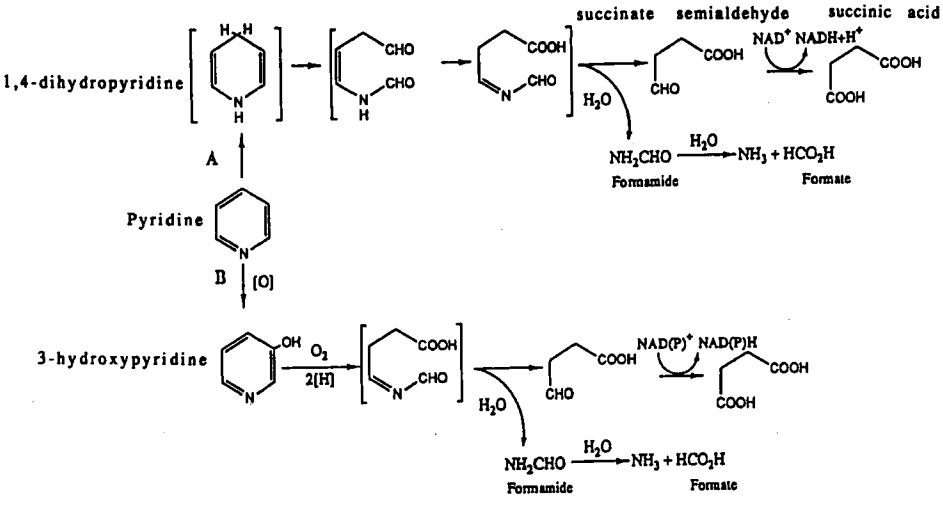


**Figure 5** Biodegradation pathways of pyridine by soil bacteria *Bacillus* 4 and *Nocardia* KM-2 (adapted from refs. 9 and 13).

Ronen and Bollag[19] investigated the biodegradation of pyridine under denitrifying conditions. A denitrifying bacterium identified as *Alcaligenes* sp. was isolated from pyridine-polluted soil. The bacterium mineralized pyridine rapidly (Figure 6) as determined by the production of $^{14}CO_2$ from $^{14}C$-labeled pyridine. The mineralization was directly linked to the reduction of nitrate to nitrogen gas. A culture of *Alcaligenes* sp. was used to enhance mineralization of pyridine in a polluted sediment[20] (Figure 7).

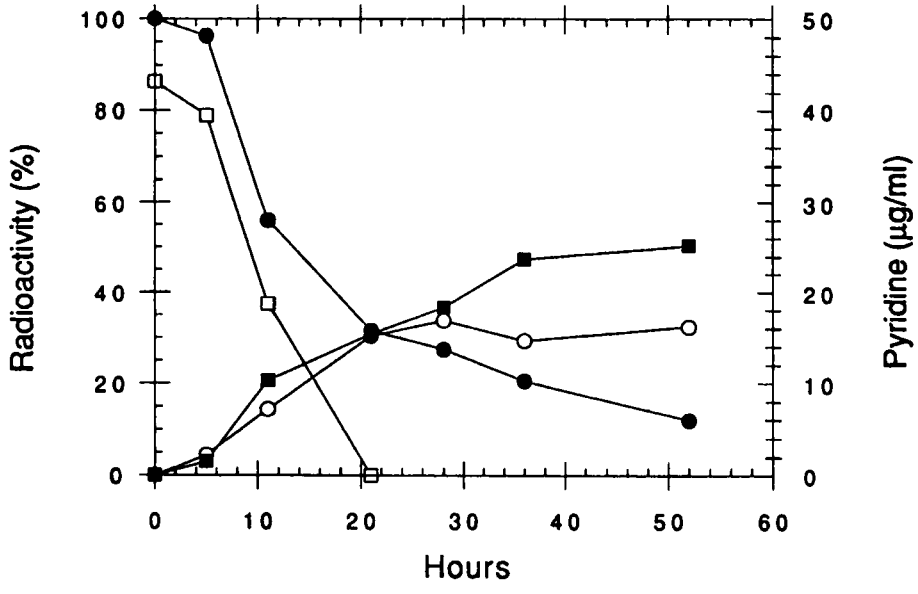


**Figure 6** Mineralization of $^{14}$C-pyridine by cultures of the isolated bacterium. The recovery of $^{14}$C-ranged between 90.5 and 103.9% of applied $^{14}$C (adapted from ref. 19).
- □ - Pyridine - ■ - $^{14}CO_2$ - ○ - $^{14}$C in biomass - ● - $^{14}$C Remaining in the medium

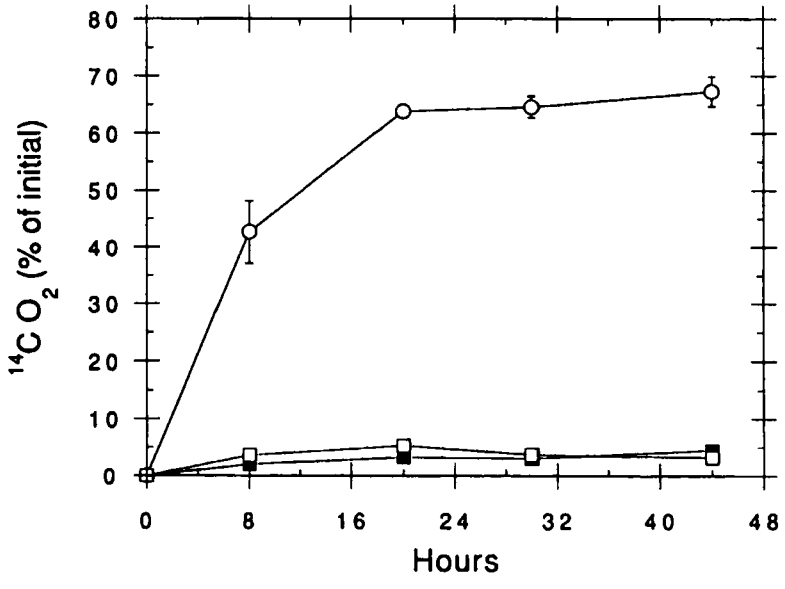


**Figure 7** Mineralization of $^{14}$C-pyridine in subsurface sediment that was amended with nitrate and incubated under anaerobic conditions at 28°C (adapted from ref. 20).
- ○ - Inoculated with the *Alcaligenes* sp. - ■ - Uninoculated - □ - Control (0.5% $NaN_3$)

Under anaerobic conditions, the introduced bacteria retained the ability to efficiently degrade pyridine. During biodegradation of pyridine by the *Alcaligenes* sp., no intermediate products accumulated. Thus, this bacterium was able to decontaminate the pyridine-polluted sediment producing $CO_2$ and biomass.

In order to evaluate the efficacy of biological treatment for the clean-up of pyridine-polluted subsurface sediments versus other decontamination methods (e.g. chemical oxidation), mineralization of pyridine by the *Alcaligenes* sp. was compared with oxidation of the compound by Fenton's reagent[21] (Figure 8). The bacterial treatment was found to be far more efficient in mineralizing pyridine in slurries of contaminated subsurface and groundwater.

## CONCLUSIONS

In the soil, biodegradation of pyridine and pyridine derivatives is mainly mediated by aerobic microorganisms. In the subsurface environment, however, biodegradation of these compounds is oxygen-limited. Recent evidence suggests that anaerobic metabolism of pyridines appears possible. The ability of denitrifying bacteria to degrade pyridine may have implications for bioremediation of polluted aquifers. Moreover, the ability of sulfate-reducing cultures derived from the subsurface suggests that biotransformation of picolines can happen without the presence of molecular oxygen. Determination of the exact metabolic pathways of these compounds is still needed.

Pyridine biodegradation by aerobic microorganisms has been extensively studied. Unlike its homocyclic analog benzene, pyridine is reduced and not hydroxylated before



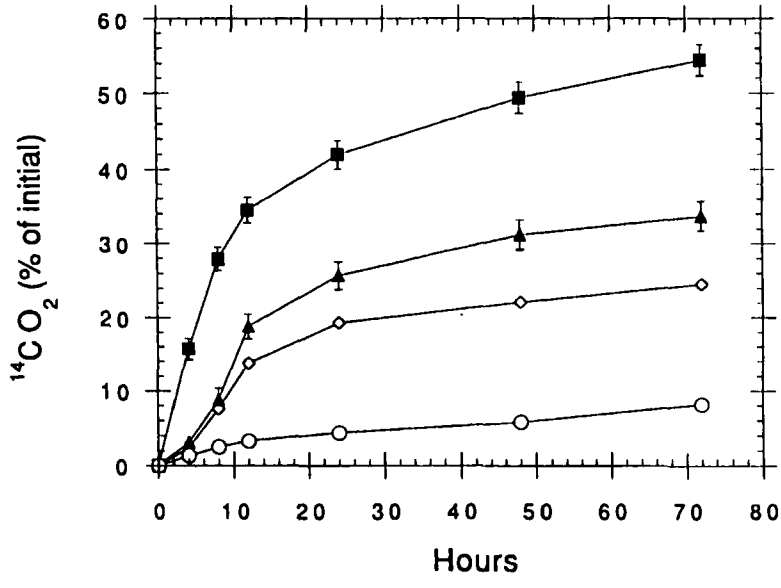


**Figure 8** Mineralization of $^{14}$C-pyridine in slurries of groundwater and subsurface sediment treated with (adapted from ref. 21):
- □ - *Alcaligenes* sp. - ▲ - Fenton's reagent - ◇ - $H_2O_2$ only - ○ - Pyridine only

ring cleavage. Furthermore, degradation of alkylpyridines involves several unusual reactions, such as *N*-oxidation and ring reduction, in addition to ring hydroxylation and alkyl group hydroxylation reactions. A variety of aerobic microorganisms, including gram-negative and gram-positive bacteria, fungi, streptomyces, and yeast are able to metabolize pyridine compounds. Bacteria usually mineralize the compounds while fungal metabolism results in partial biotransformation of the chemicals.

Pyridine could be degraded under anaerobic conditions by mixed cultures of denitrifying, sulfate-reducing, and methanogenic bacteria. Although a denitrifying *Alcaligenes* sp. mineralizes pyridine rapidly, there is still very little information on the identity of other pyridine-degrading anaerobic microorganisms. Additional studies should focus on this topic.

In summary, pyridine and its derivatives are degraded by aerobic soil microorganisms and, to a lesser extent, by anaerobic microorganisms. The information presented suggests that bioremediation of pyridine-polluted soil, subsurface sediment, and groundwater is a feasible treatment.

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