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Project No. DE-FG02-87ER60556

A.) Progress Report for the Current Budget Period

(July 1, 1987 - June 30, 1989)

1.) Comparison of the Activity of Subsurface and Surface Microorganisms and Their Anaerobic Transformation of Heterocyclic Compounds

Our interest in this research was mainly to compare the physiological characteristics of microorganisms derived from subsurface and surface environments and their ability to transform heterocyclic aromatic chemicals. We selected until now essentially indole and pyridine compounds as representatives of heterocyclic compounds. The samples investigated originated from the subsurface drillings at the Savannah River Plant, from surface samples in Pennsylvania, and from municipal sewage of State College, Pennsylvania.

At different physiological conditions (aerobic, denitrifying, sulfate-reducing or methanogenic), different groups of microorganisms are active. Not only the thermodynamics of microbial physiology vary, but different metabolic pathways are used by the various types of microbial processes. Therefore, it was important to determine under which physiological conditions a compound was metabolized, and to clarify the metabolic conditions under which intermediate(s) were produced.

a.) Experiments with indole

In studies with indole as substrate our results contributed to the concept that anaerobic biodegradation of an organic chemical is strongly influenced by the prevailing metabolic regimen (methanogenesis or denitrification) and several environmental factors, but apparently not by the inoculum source. Under methanogenic conditions, an inverse

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relationship between inoculum concentration and oxindole accumulation was found with sewage sludge and Carlisle muck. Also, temporary stoichiometric conversion of indole to oxindole was effected by both Carlisle muck and freshwater sediments. Moreover, by the manipulation of temperature and inoculum concentration, the trend toward conversion of indole to oxindole by sewage sludge was found to resemble that of freshwater sediments. If neither temperature nor inoculum concentration influenced oxindole accumulation, unique metabolic characteristics of the two different microbial communities would be implicated.

We also observed a uniform pattern of indole metabolism under denitrifying conditions, regardless of the source of inoculum. With both Carlisle muck and sewage sludge, oxindole never accumulated at high levels. However, traces of oxindole were detected during the period of rapid indole disappearance, and added oxindole was metabolized without a lag period. These two observations suggest that oxindole is an intermediate metabolite of indole during its biodegradation under denitrifying conditions. Accordingly, the absence of oxindole accumulation during indole metabolism under denitrifying conditions could be explained by a rate of oxindole disappearance that equals the rate of its appearance. Data supporting this hypothesis are shown in Fig. 1, in which oxindole loss actually exceeded the rate of disappearance of its likely precursor, indole.

Regardless of temperature, inoculum source, or inoculum concentration, oxindole always accumulated under methanogenic conditions, but never under denitrifying conditions. Thus, the type of physiological metabolism (as defined by final electron acceptor) had a dominant effect on oxindole accumulation during indole transformation.

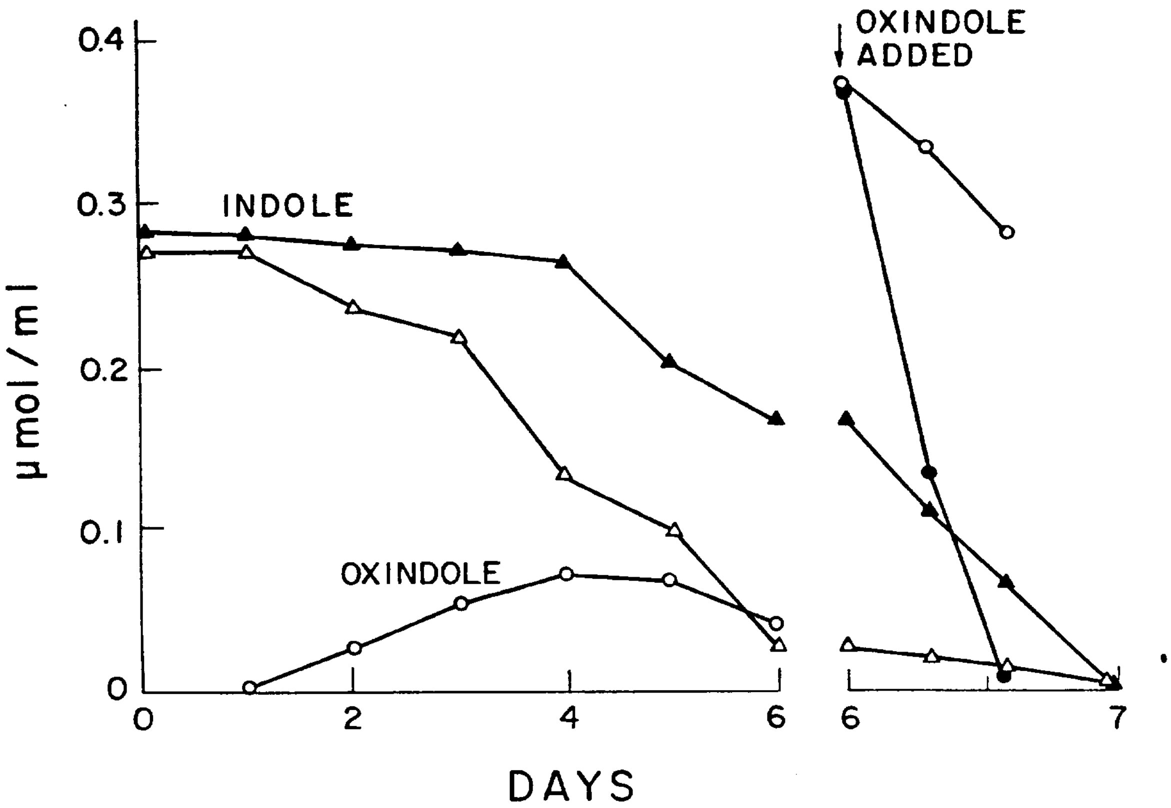

Fig. 1. Transformation of indole and oxindole by 9% digested sludge under methanogenic (○, △) and denitrifying (●, ▲) conditions.

The mechanism which shifted relative rates of oxindole production and elimination is uncertain, but we speculate that community structure, species diversity, and the size and activity of microbiological populations (which varied with temperature, concentration of inocula, and final electron acceptor) caused the observed patterns of oxindole accumulation.

We also sought to define early steps of denitrifying indole metabolism using mixed cultures from an environmental sample (sewage sludge). Denitrifying conditions were also of interest because of the ubiquity and metabolic potential of denitrifying microorganisms. Mixed cultures were used because of their ecological and environmental relevance; this approach has been employed extensively in documenting the metabolism of pesticides in soil and can complement investigations using single microorganisms.

Based upon the structures of chemicals identified and their sequential occurrence, we postulate a metabolic pathway for the early stages of indole metaboilism under denitrifying conditions (Fig. 2). Indole is hydroxylated first at the 2-position to form oxindole and subsequently at the 3-position to form isatin. Next, we propose addition of two hydroxyl groups to yield isatoic acid. The inclusion of isatoic acid in this scheme seems reasonable from a structural standpoint, but is purely speculative because our attempts to isolate isatoic acid were unsuccessful. Isatoic acid is chemically unstable and spontaneously decarboxylates to form anthranilic acid. However, isatoic acid has also been proposed as an intermediate metabolite during aerobic metabolism of indole. The physiological significance of dioxindole in this metabolic pathway is unknown. Dioxindole is formed readily from isatin in the presence of reducing agents such as sodium

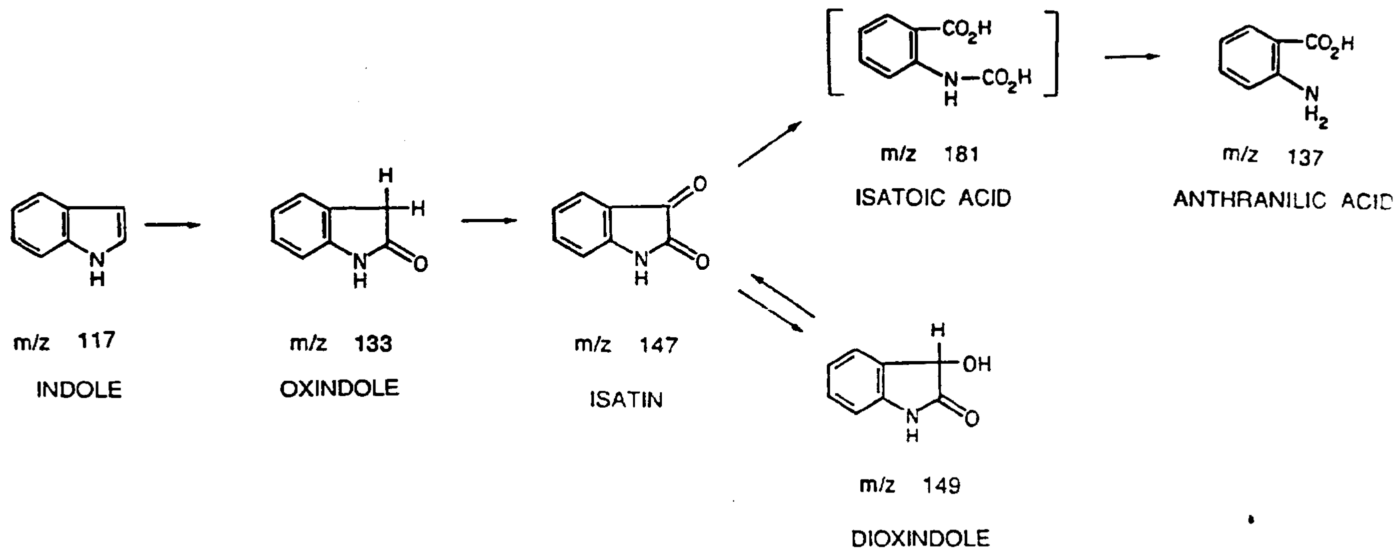

Fig. 2. Proposed metabolic pathway for the early stages of indole transformation by denitrifying sewage-sludge microorganisms.

sulfite and sodium hydrosulfite. These or other reducing agents were very probably present in the sewage-sludge inoculum: therefore, the mechanism by which dioxindole was formed (chemical versus microbiological) is uncertain.

b.) Experiments with pyridine and derivatives

Pyridine was transformed by subsurface organisms under aerobic conditions similarly as organisms isolated from the surface. Since in most subsurface environments oxygen as an electron acceptor may not be present, we concentrated our studies on anaerobic environments (nitrate-reduction, sulfate-reduction and fermentation).

Most samples from the subsurface are using lactate under sulfate-reducing conditions. This behavior is similar to samples which originate from anaerobic surface environments (most sulfate-reducing organisms can use lactate as substrate). When ^{35}sulfate is added to these samples, the electron acceptor is reduced to ^{35}sulfide. Pyridine on the other side was not degraded under sulfate-reducing conditions.

Under methanogenic conditions (i.e. without additional electron acceptor) no transformation occurred with samples from the subsurface. It is not surprising for the tested heterocyclic compounds, because organisms from the surface also degrade these compounds at a slow rate. Without an electron acceptor only 3-hydroxypyridine was degradable.

We have tested several heterocyclic compounds such as pyridine, 2-, 3- and 4-hydroxypyridine, and methylated pyridines such as 2-, 3-, and 4-picoline, 2,4-, 2,6-, 3,4- and 3,5-lutidine under different physiological conditions with surface organisms and with pure cultures which had been isolated from subsurface environments. From the 10 pure subsurface cultures which we obtained, 6 had been able to grow not only under aerobic conditions, but also as a denitrifier in a nitrate broth medium. This observation showed that the organisms from the subsurface have a similar behavior like microorganisms, which had been isolated from the surface environment. Some of these bacteria are also able to use nitrate as an electron acceptor, when no oxygen is available. Unfortunately, none of the pure cultures had been able to degrade any of the tested pyridine, hydroxypyridines or methylated pyridines.

Because the metabolic pathways vary with different types of microbial processes, toxic organic compounds may be metabolized to various intermediates under changed physiological conditions. Even if no toxic intermediates are produced, it is important to understand how different physioligical conditions affect the susceptibility of organic compounds to microbial attack. Therefore, we made studies with cultures obtained from the surface environment.

Under aerobic conditions pyridine and all tested monohydroxypyridines were degradable. Under nitrate- and sulfate-reducing conditions only pyridine, 3-hydroxypyridine and 3- and 4-picoline were degradable. The pyridine-degrading culture was also able to transform 3-hydroxypyridine. The metabolism of 3-hydroxypyridine by this culture is interesting, because all isolated organisms which degrade pyridine in the presence of oxygen were not able to metabolize any monohydroxypyridines. In spite of different tests with labelled pyridine, we had never been able to measure significant amounts of ^{14}C-3-hydroxypyridine. It is therefore possible that the culture, which degrades pyridine and 3-hydroxypyridine is using different pathways for the transformation of these two molecules. Because these substances were also toxic for microorganisms in higher concentrations, only low amounts (20 mg/l) had been provided. Therefore, the concentrations of intermediate products formed during the transformations of the substrates were low, which makes the identification of products difficult.

In many biological transformations one or more of the carbon atoms of the substrates are oxidized to carboxyl groups. All these products which contain carboxyl groups can be detected in low concentrations after derivatization with 2-nitrophenylhydrazine. During the degradation of 3-hydroxypyridine we were able to detect low concentrations of acetate as an intermediary product. Acetate can be degraded under aerobic as well as anaerobic conditions by a large number of microorganisms. Therefore, we can expect that 3-hydroxypyridine can be metabolized by anaerobic bacteria (when nitrate or sulfate is available) in groundwater contaminated with this compound.

Further we succeeded in isolating new cultures which are able to degrade methylated pyridines under different physiological conditions. When oxygen was present. 2-picoline (2-methylpyridine) and 3-picoline were relatively easy to degrade. 2-Picoline or 3-picoline (30 mg/L) were converted by cultures within 7 to 12 days. We always observed that the metabolism of 2-methylpyridine was faster than the degradation of 3-methylpyridine. 4-Picoline, four different dimethylpyridines and 2,4,6-trimethylpyridine were not converted under aerobic conditions in the first 6 months.

Under anaerobic conditions 3- and 4-picoline can be degraded when either nitrate or sulfate is present. The metabolism of these two picolines is very slow. The metabolism of 20 mg/L of these compounds takes about 1 to 2 months. Because these cultures were also able to degrade pyridine, it is possible that the degradation of these methylated pyridines under anaerobic conditions starts with a demethylation reaction. It appears that pyridine is the first intermediate product in the anaerobic degradation of 3- and 4-picoline. In this case the pathway in the degradation of methylated pyridines and pyridine may be the same under various anaerobic conditions. 2-Picoline, which was degraded relatively easily in the presence of oxygen, was not biotransformed under anaerobic conditions. It is possible that steric hindrance blocks the degradation. As already mentioned, we suspect that 3- and 4-picoline are demethylated to pyridine under anaerobic conditions. A further indication for this hypothesis is the transformation of 2,4,6-trimethylpyridine (1,4,6-collidine). This substrate is converted by an active pyridine-degrading culture to 2-methylpyridine (1-picoline), which accumulated as an endproduct in the culture media. The release of the methylgroup (in <u>para</u>-position) is not surprising, because this culture was also able to degrade 4-methylpyridine. The accumulation of 2-picoline during the degradation of 2,4,6-collidine is not unexpected, because these organisms are not able to degrade 2-methylpyridine. Presently, however, it is not clear why only one of the two methylgroups in <u>ortho</u>-position can be cleaved.

2.) Determination of Factors Which Affect Anaerobic Microbial Activity in the Deep Subsurface (Fourth Hole of the Savannah River Plant)

The objective of this part of our research was to verify: (1) the presence and diversity (aerobic to strictly anaerobic) of *in situ* microorganisms depending on depth and geologic setting, and (2) their metabolic potential especially in relation to heterocyclic molecules like indole and pyridine.

Earlier studies with sediment samples from Savannah River Plant demonstrated the presence of microorganisms in sediments up to depths of 262 m (Madsen and Bollag, 1989). However, there appears to be no relationship between sediment depth and microbial activity in the depth subsurface as even sediments from depths of 463 and 526 m transformed glucose at rates comparable to the surface while little transformation was observed in sediments from 56, 71, 303, and 324 m. The rate of glucose transformation observed at various sediment depths appeared to be in agreement with the total colony forming units reported at those depths.

Indole transformation demonstrated similarities to glucose mineralization but the rate of transformation of indole was not comparable to glucose at various sediment depths. Most of the sediments which exhibited little glucose mineralization also had similar or no transformation of indole even after 4 weeks of incubation. However, the transformation of indole in about 80% of the sediments tested is evidence of the metabolic versatility of the microorganisms present at these depths, considering the structural complexity of the molecule.

Pyridine transformation under aerobic conditions was very slow and maximum transformation was observed only in sediments which were actively metabolizing glucose and indole.

Sulfate reduction could be detected in 25% of the subsurface sediments analyzed (after 4 weeks of incubation) and only 2 appeared to be carbon limited as was evident on amendment with lactate, a conventional substrate for sulfate-reducing bacteria. The assumption that deeper sediment layers should be much more anoxic and may exhibit more anaerobic microbial activity than those near the surface was not found to be entirely correct for samples from the deep subsurface. The only example was from 290 m where no transformation of indole was observed under aerobic conditions but significant transformation was detected under sulfate-reducing conditions suggesting predominance of anaerobic metabolism in this layer.

The ability of microorganisms in the deep subsurface sediments to actively metabolize not only glucose but also heterocyclic structures like indole and pyridine is encouraging considering the remoteness of these sediments from the surface environment. Our data suggest that deep terrestrial microorganisms vary considerably in density and activity from one depth to another. Ih addition, microorganisms at various depths not only respond differently to contaminants or nutrient amendments but exhibit physiological activity comparable to the surface environment. The observations on high aerobic activity (even at depths of 526 m or 1725 feet) against the expected anaerobic activity even after long incubations (detection of sulfate reduction and methanogenesis in only 25 and 50% of the sediments, respectively) at these depths are not easy to explain with the state of our current knowledge on deep subsurface, perhaps more geochemical and hydrological data would help in better understanding of microbial ecology of the terrestrial deep subsurface.

B.) List of Publications and Papers Resulting from

DOE Supported Grant No. DE-FG02-87ER60556

1.) Publications

Berry, D. F., E. L. Madsen and J.-M. Bollag
Conversion of indole to oxindole under methanogenic conditions
Appl. Environ. Microbiol. 53, 180-182 (1987)

Berry, D. F., A. J. Francis and J.-M. Bollag
Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions
Microbiol. Rev. 51, 43-59 (1987)

Madsen, E. L., A. J. Francis and J.-M. Bollag
Environmental factors affecting indole metabolism under anaerobic conditions
Appl. Environ. Microbiol. 54, 74-78 (1988)

Madsen, E. L. and J.-M. Bollag
Pathway of indole metabolism by a denitrifying microbial community
Arch. Microbiol. 151, 71-76 (1989)

Bollag, J.-M. and J. Czaban
Effect of microorganisms on extractability of Cd from soil with NaOH and DTPA
J. Soil Sci. (1989) (in press)

Madsen, E. L. and J.-M. Bollag
Aerobic and anaerobic microbial activity in deep subsurface sediments from the Savannah River Plant
Geomicrobiol. J. (1989) (in press)

Kurek, E., A. J. Francis and J.-M. Bollag
Microbial immobilization of cadmium released from CdO in soil
(to be submitted to Arch. Environ. Contam. Toxicol.) (1989)

Fliermans, C. B., et al.
Microbial life in the deep terrestrial subsurface
(to be submitted to Bioscience) (1989)

Ronen, Z. and J.-M. Bollag
Anaerobic metabolism of pyridine by a denitrifying population
(to be submitted to Appl. Environ. Microbiol.) (1989)

Shanker, R. and J.-M. Bollag
Microbial diversity and transformation of heterocyclic chemicals in deep-subsurface sediments.
(to be submitted to Arch. Microbiol.) (1989)

2.) Papers Presented at Scientific Meetings

Bollag, J.-M., P. Chanmugathas, and A. J. Francis
The effect of microorganisms on cadmium mobilization in soil
Am. Soc. Microbiol., Ann. Meeting, Atlanta, Ga. Abstract (1987)

Madsen, E. L. and J.-M. Bollag
Microbial activity in deep subsurface sediments from the Savannah River Plant
Am. Soc. Microbiol., Miami Beach, Fla. Abstract (1988)

Ronen, Z.
Degradation of pyridine by a microbial population under denitrifying conditions
Am. Soc. Microbiol., Ann. Meeting, New Orleans, La. Abstract (May 1989)

Shanker, R. and J.-M. Bollag
Transformation of indole by methanogenic and sulfate-reducing organisms.
Am. Soc. Microbiol., Ann. Meeting, New Orleans, La. Abstract (May 1989)

Kaiser, J.-P. and J.-M. Bollag
Metabolism of pyridine and pyridine derivatives under aerobic and anaerobic conditions
Am. Soc. Microbiol., Ann. Meeting, New Orleans, La. Abstract (May 1989)

Bollag, J.-M., Z. Ronen and J.-P. Kaiser
Biodegradation of pyridine and pyridine derivatives under different physiological conditions
Fifth Int. Symp. on Microbiol. Ecology, Kyoto, Japan (Aug. 27-Sept. 1, 1989)